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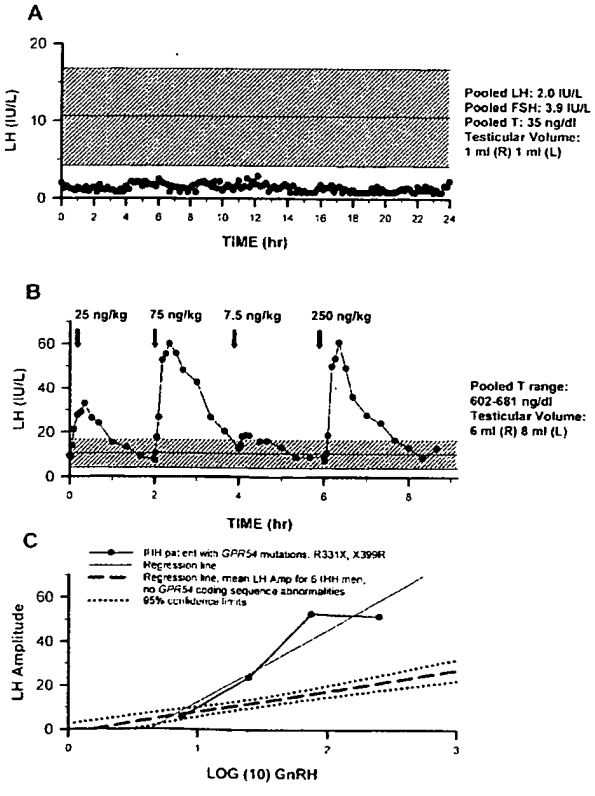

(57) Abstract: The present invention relates to the identification of compounds for diagnosing and treating a reproductive disorder or other disorders in which the suppression of gonadal steroids has therapeutic benefits. These compounds can be used to treat, for example, polycystic ovarian disease, endometriosis, uterine fibroids, and prostate cancer. Moreover, the compounds identified using the methods of the invention may be used as contraceptives or in treatments for infertility, such as *in vitro* fertilization. The invention also relates to mutant GPR54 nucleic acid molecules and polypeptides, as well as their use in diagnosis of reproductive disorders.

WO 2004/101747 A2

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IDENTIFICATION AND USE OF GPR54 AND ITS LIGANDS FOR REPRODUCTIVE DISORDERS AND CONTRACEPTION

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Background of the Invention

In general, the invention relates to screens for the discovery of compounds useful for the medical management of several reproductive disorders and contraception.

The pulsatile secretion of gonadotropin hormone releasing hormone (GnRH) from the hypothalamus generates the normal reproductive axis of boys and girls during the neonatal period (Waldhauser et al., Eur. J. Pediatr. 137:71-74, 1981), and again at puberty (Boyar et al., J. Clin. Invest. 54:609-618, 1974), whereas a marked decrease in the amplitude of its secretion causes the quiescence of reproductive activity characteristic of childhood (Ross et al., J. Clin. Endocrinol. Metab. 57:288-293, 1983; Wu et al., J. Clin. Endocrinol. Metab. 70:629-637, 1990; and Dunkel et al., J. Clin. Endocrinol. Metab. 74:890-897, 1992).

Abnormalities in secretion of GnRH, or impairment of an individual's response to GnRH, can lead to a variety of disorders including idiopathic hypogonadotropic hypogonadism ("IHH"), microphallus, i.e., a stretched penile length that is less than 2.5 cm at birth, and hypospadius, i.e., the incomplete fusion of the penile urethra. In addition, abnormalities in the secretion of GnRH or in a person's response to GnRH can result in hypogonadotropic hypogonadism in men and women, hypothalamic amenorrhea, e.g., the absence of the normal initiation of menses or the cessation of menses for at least 3 months in a woman with previously normal menstrual cycles, delayed puberty, and possibly polycystic ovarian disease. In addition, it is possible to induce hypogonadotropic hypogonadism with GnRH

analogues for therapeutic purposes in the settings of central precocious puberty, prostate cancer, endometriosis, and uterine fibroids, as well as suppressing the gonad for purposes of contraception and treatment of polycystic ovarian disease.

Furthermore, GnRH and/or its analogues which produce hypogonadotropic hypogonadism also may be used in preparation for *in vitro* fertilization (IVF). Fifteen percent of all couples have difficulty with conception. In general, IVF is a means to achieve pregnancy using the following steps: (1) superovulation of the female partner, (2) retrieval of the mature follicles, (3) joining of the eggs and sperm for conception *in vitro*, (4) culturing and support of the embryos, and (5) transfer of embryos to the uterus. Superovulation is often achieved by first administering a GnRH agonist for two weeks to inhibit endogenous gonadotropin production (i.e. induce hypogonadotropic hypogonadism) prior to induction of ovulation, thereby maximizing control of the menstrual cycle. Alternatively, GnRH antagonists can result in more rapid desensitization and hence production of a hypogonadotropic state than GnRH agonists, and may reduce the risk of ovarian hyperstimulation syndrome (Paulson and Marrs, Curr. Probl. Obstet. Gynecol. Infert. 10:497, 1986; and Inany and Aboulghar, Cochrane Database Syst. Rev. 4:CD001750, 2001).

IHH is a condition in which puberty fails to occur in the face of low/normal gonadotropins and the absence of any anatomic or functional cause. As nearly all patients with IHH respond to exogenous GnRH, the defect in this disorder is likely at the level of the secretion or action of the hypothalamic peptide GnRH. Typically, IHH becomes apparent in late adolescence with failure of sexual development heralded by the absence of the normal appearance of secondary sex characteristics. The condition is characterized by (1) complete or partial absence of endogenous GnRH-induced LH pulsations (Boyar et al., J. Clin. Endocrinol. Metab. 43:1268-1275, 1976; Crowley et al., Rec. Prog. Horm. Res. 41:473-531, 1985; Santoro et al., Endocr. Rev. 7:11-23, 1986; Spratt et al., J. Clin. Endocrinol. Metab. 64:283-291, 1987; and Pitteloud et al., J. Clin. Endocrinol. Metab. 87:152-160, 2002), (2) a lack of any anatomic cause on radiographic imaging of the hypothalamus and pituitary (Whitcomb and Crowley, "Male Hypogonadotropic Hypogonadism." In: Veldhuis, editor, Endocrinology and Metabolism Clinics of North America, Philadelphia: WB Saunders Co. 125-143,

1993; and Ross et al., J. Clin. Endocrinol. Metab. 57:288-293, 1983), (3) normal baseline and reserve testing of the remaining hypothalamic-pituitary axes, and (4) a generally normal response to physiologic replacement with exogenous GnRH, localizing the defect to an abnormality of GnRH synthesis, secretion, or action (Santoro et al., Endocr. Rev. 7:11-23, 1986; Crowley and McArthur, J. Clin. Endocrinol. Metab. 51:173-175, 1980; and Hoffman and Crowley, N. Engl. J. Med. 307:1237-1241, 1982).

Despite this well documented genetic heterogeneity, the discovery of new genes for IHH has remained at a standstill as the hallmarks of hypogonadotropic hypogonadism--disease rarity, reproductive "lethality," phenotypic diversity, and genetic heterogeneity -- represent formidable obstacles to genetic analysis. Consequently, there is a need to identify genes that are mutated in IHH as well as other reproductive and sexual maturation disorders.

Summary of the Invention

The present invention relates to methods that can be used for the identification of compounds that can affect GnRH secretion or action - either to decrease its secretion and cause hypogonadotropic hypogonadism as a therapy for various medical conditions such as prostate cancer, endometriosis, central precocious puberty, IVF, uterine fibroids, polycystic ovarian disease, and for contraception or restore normal pulsatile GnRH secretion in disorders or conditions in which it is abnormal. Because GPR54's normal function is to modulate and support GnRH secretion and mutations in this receptor cause a form of hypogonadotropic hypogonadism, antagonist compounds which decrease a biological activity of a GPR54 polypeptide will result in a similar hypogonadotropic state, a circumstance that has great therapeutic value for treating central precocious puberty, prostate cancer, endometriosis, uterine fibroids, as well as in methods of contraception, and as a preparation for *in vitro* fertilization. Moreover, compounds which increase a biological activity of a GPR54 polypeptide identified using the methods of the invention, i.e., by screening for agonist activity in a GPR54-based methodology, may be used in treatments for infertility, such as amenorrhea, and for the treatment of IHH, and polycystic ovarian disease. The

methods of the present invention use a GPR54 receptor, the biological activity of which can regulate GnRH secretion, as a technique for discovering both antagonists and agonists for this receptor to modify endogenous GnRH secretion. Further, mutations in a nucleic acid sequence encoding a GPR54 polypeptide can result in a reproductive disorder such as hypogonadotropic hypogonadism.

Accordingly, the first aspect of the invention features a method of identifying a candidate compound for suppressing endogenous GnRH secretion or action. This method involves contacting a GPR54 polypeptide with a test compound and assaying a biological activity of the GPR54 polypeptide, where a decrease in the biological activity, relative to a control not contacted with the test compound, identifies the test compound as a candidate compound for suppressing endogenous GnRH secretion or action. In a desirable embodiment of the first aspect of the invention, the GPR54 polypeptide is contacted with the test compound in the presence of a kisspeptin polypeptide and the control is in the presence of a kisspeptin polypeptide.

In a related aspect, the invention features another method of identifying a candidate compound for suppressing endogenous GnRH secretion or action. This method involves contacting a cell with a test compound and metastin and assaying for a decrease of GnRH secretion or action, relative to a control cell in the presence of a kisspeptin polypeptide and not contacted with the test compound, where a decrease in GnRH secretion or action identifies the test compound as a candidate compound for suppressing endogenous GnRH secretion or action. In desirable embodiments, the kisspeptin polypeptide includes amino acids 68-121 or 112-121 of human kisspeptin-1.

In another related aspect, the invention features a method of identifying a candidate compound for treating a disorder selected from the group consisting of idiopathic hypogonadotropic hypogonadism, amenorrhea, delayed puberty, and polycystic ovarian disease. This method involves contacting a GPR54 polypeptide with a test compound and assaying a biological activity of the GPR54 polypeptide, wherein an increase in the biological activity, relative to a control not contacted with

the test compound, identifies the test compound as a candidate compound for treating idiopathic hypogonadotropic hypogonadism, amenorrhea, delayed puberty, or polycystic ovarian disease.

The invention further features a method of identifying a candidate compound for use in treating infertility, in *in vitro* fertilization or contraception. This method involves contacting a GPR54 polypeptide with a test compound and assaying a biological activity of the GPR54 polypeptide, where an alteration of the biological activity, relative to a control not contacted with the test compound, identifies the test compound as a candidate compound for use in treating infertility, in *in vitro* fertilization or contraception. Candidate compounds identified using this method may also be used to treat central precocious puberty, prostate cancer, endometriosis, and uterine fibroids. In a desirable embodiment of this method, the candidate compound induces a state of hypogonadotropic hypogonadism in a patient. In other desirable embodiments of this method, the alteration is a decrease in the biological activity, e.g., by means of an antagonist to the normal function of GPR54 whose biological role is to support endogenous GnRH secretion. In further desirable embodiments, the alteration is an increase in the biological activity, e.g., by means of an agonist to restore normal GnRH secretion or action.

In a second aspect, the invention features a method for treating a reproductive disorder selected from the group consisting of central precocious puberty, polycystic ovarian disease, endometriosis, and uterine fibroids, in a mammal. This method involves administering an effective amount of a compound that decreases a biological activity of a GPR54 polypeptide to the mammal. In a desirable embodiment of this method, the candidate compound induces a state of hypogonadotropic hypogonadism in a mammal.

In a related aspect, the invention features a method for treating a reproductive disorder selected from the group consisting of idiopathic hypogonadotropic hypogonadism, amenorrhea, and polycystic ovarian disease, in a mammal. This method involves administering an effective amount of a compound that increases a biological activity of a GPR54 polypeptide to the mammal.

In a third aspect, the invention features a method for contraception in a mammal. This method involves administering an effective amount of a compound that alters a biological activity of a GPR54 polypeptide to the mammal. In a desirable embodiment of this aspect of the invention, method further involves the administration of a steroid to the mammal. In additional embodiments, the steroid may be estrogen and/or progesterone in a female and testosterone in a male.

In a fourth aspect, the invention features a method for treating infertility in a mammal. This method involves administering an effective amount of a compound that alters a biological activity of a GPR54 polypeptide the mammal.

In desirable embodiments of the third and fourth aspect of the invention, the compound decreases a biological activity of a GPR54 polypeptide. In other desirable embodiments of these aspects, the compound increases a biological activity of a GPR54 polypeptide. In additional desirable embodiments of these methods, the mammal is a human.

In other desirable embodiments of these methods, the compound decreases a biological activity of a GPR54 polypeptide. In additional desirable embodiments, the compound increases a biological activity of a GPR54 polypeptide. In yet further desirable embodiments, the mammal to be treated is a human, monkey, dog, cat, horse, cow, pig, deer, or a rodent, e.g., a mouse or rat.

In a further aspect, the invention features a method of altering the internal levels of gonadal sex steroids in a mammal with a proliferative disorder, such as a cancer. This method involves contacting a mammal with a compound that decreases a biological activity of a GPR54 polypeptide to induce a hypogonadotropic state. In desirable embodiments, this method may be used to treat prostate or breast cancer, as well as any other malignancy that is affected by the internal level of a gonadal sex steroid. In other desirable embodiments, the gonadal sex steroid is testosterone, androstenedione, estradiol, DHEA-sulfate, prolactin, and di-hydrotestosterone in males, and testosterone, androstenedione, DHEA-sulfate, prolactin, and di-hydrotestosterone, estradiol (e2), estrone (e1), estriol (e3), and progesterone, in females.

In a fifth aspect, the invention features a method of diagnosing a reproductive disorder, e.g., delayed and/or central precocious puberty, idiopathic hypogonadotropic hypogonadism, amenorrhea, or polycystic ovarian disease in a mammal. This method involves determining whether a mammal has an alteration in a nucleic acid sequence containing a GPR54 nucleic acid molecule, or a fragment thereof, relative to a control sequence, where the presence of the alteration is indicative that the mammal has or has a propensity for developing the reproductive disorder. In a desirable embodiment of this method, the mammal is a human. In additional desirable embodiments of this aspect, an alteration results in an amino acid change at a position corresponding to amino acid 148, 331, or 399 of a human GPR54 amino acid sequence.

In a related aspect, the invention features another method of diagnosing a reproductive disorder in a mammal. This method involves determining whether the mammal has an alteration in the expression or biological activity of a GPR54 nucleic acid molecule or GPR54 amino acid molecule, or a fragment thereof, relative to a control nucleic acid sequence or amino acid sequence, where the alteration of expression or biological activity is indicative that the mammal has or has a propensity for developing a reproductive disorder. In a desirable embodiment of this aspect, the alteration is an alteration in the expression level of a GPR54 messenger RNA molecule. Desirably, the expression level is determined using reverse transcriptase polymerase chain reaction (RT PCR). In another desirable embodiment of this aspect, the alteration is an alteration in a biological activity of a GPR54 polypeptide. Desirably, the biological activity involves an alteration in the level of inositol phosphate production. In a further desirable embodiment of this method, the mammal is a human.

In the sixth aspect, the invention features an isolated GPR54 nucleic acid sequence encoding a polypeptide containing an amino acid change at a position corresponding to amino acid 148 of a human GPR54 amino acid sequence. In desirable embodiments of this aspect, the nucleic acid sequence encodes a Serine at position 148 of a human GPR54 amino acid sequence and/or the nucleic acid sequence contains a T to C alteration at position 443 of a human GPR54 nucleic acid sequence.

In the seventh aspect, the invention features an isolated GPR54 nucleic acid sequence encoding a polypeptide containing an amino acid change at a position corresponding to amino acid 331 of a human GPR54 amino acid sequence. In desirable embodiments of this aspect, the nucleic acid sequence encodes a stop codon at position 331 of a human GPR54 amino acid sequence and/or the nucleic acid sequence contains a C to T alteration at position 991 of a human GPR54 nucleic acid sequence.

In the eighth aspect, the invention features an isolated GPR54 nucleic acid sequence encoding a polypeptide containing an amino acid change at a position corresponding to amino acid 399 of a human GPR54 amino acid sequence. In desirable embodiments of this aspect, the nucleic acid sequence encodes an Arginine at position 399 of a human GPR54 nucleic acid sequence and/or the nucleic acid sequence contains a T to A alteration at position 1195 of a human GPR54 nucleic acid sequence.

Desirably, nucleic acid sequence of the sixth, seventh, or eighth aspect of the invention is included in a vector, such as an expression vector. Further, this vector may be contained in a cell, e.g., a mammalian cell.

The ninth aspect of the invention features an isolated polypeptide including a human GPR54 amino acid sequence which contains an amino acid substitution at position 148, 331, or 399, relative to a polypeptide encoded by GenBank Accession No. AY029541, AF343725, NM_032551, or AY253981.

Definitions

As used herein, by a "GPR54 nucleic acid" is meant a nucleic acid molecule that encodes a polypeptide that is substantially identical or is identical to the polypeptide encoded by GenBank Accession Number AY029541, AF343725, NM_032551, or AY253981, or a fragment thereof. Desirably, this nucleic acid sequence encodes a polypeptide that has a GPR54 biological activity.

As used herein, by a "GPR54 polypeptide" or a "GPR54 protein" is meant an amino acid sequence that is substantially identical or is identical to the polypeptide

encoded by GenBank Accession Number AY029541, AF343725, NM_032551, or AY253981, or a fragment thereof. Desirably, a GPR54 polypeptide or GPR54 protein has a GPR54 biological activity.

By a "GPR54 biological activity," as used herein, is meant a GPR54-dependent alteration in intracellular calcium release, a change in the phosphorylation state of a polypeptide in a signaling pathway regulated by GPR54, a GPR54-dependent alteration in arachidonic acid release from a cell, a GPR54-dependent alteration in phosphatidylinositol turnover, or an alteration in endogenous GnRH secretion. A GPR54 biological activity may be regulated by a compound, e.g., a peptide or a small molecule, that binds to GPR54 or that alters the expression of a GPR54 nucleic acid molecule or polypeptide. Further, compounds that alter the activity or expression of components of a GPR54 signaling pathway may also alter a GPR54 biological activity. Guidance for assaying protein interactions, function, and expression may be found in, for example, Ausubel et al. (*Current Protocols in Molecular Biology*, Wiley Interscience, New York, 2001).

By "altering a GPR54 biological activity," as used herein, is meant a decrease or an increase in a GPR54 biological activity, relative to a control. Preferably, the decrease in GPR54 biological activity is at least 20%, 40%, 50%, or 75% below that of an untreated control as measured by any standard assay known in the art, including the assays described herein. More desirably, the biological activity of a GPR54 polypeptide is decreased by 80%, 90%, 95%, or even 100% below that of an untreated control. Alternatively, an alteration in GPR54 biological activity may be an increase in GPR54 biological activity that is at least 20%, 40%, 50%, or 75% above that of an untreated control as measured by any standard assay known in the art, including the assays described herein. More desirably, the increase in GPR54 biological activity is at least 80%, 90%, 95%, or even 100% above that of an untreated control. Such responses can be monitored using, for example, calcium release assays, determining the phosphorylation state of a polypeptide in a signaling pathway regulated by GPR54, arachidonic acid release assays, phosphatidylinositol turnover assays, and an alteration in endogenous GnRH secretion.

By an "alteration in endogenous GnRH secretion," as used herein, is meant a decrease or an increase in a GnRH secretion from a cell, relative to a control. Preferably, the decrease in GnRH secretion is at least 20%, 40%, 50%, or 75% below that of an untreated control as measured by any standard assay known in the art, including the assays described herein. More desirably, secretion of GnRH is decreased by 80%, 90%, 95%, or even 100% below that of an untreated control. Alternatively, an alteration GnRH secretion may be an increase in GnRH secretion that is at least 20%, 40%, 50%, or 75% above that of an untreated control as measured by any standard assay known in the art, including the assays described herein. More desirably, the increase in GnRH secretion is at least 80%, 90%, 95%, or even 100% above that of an untreated control. Such responses can be monitored, for example, by exposing a cell expressing a GPR54 polypeptide to a test sample and assaying the biological activity of the GPR54 polypeptide relative to a control not exposed to the test sample, or by exposing a cell expressing a GnRH-regulated reporter gene, e.g., luciferase, to a test sample and assaying reporter gene expression relative to a control cell not exposed to the test sample.

By a "kisspeptin polypeptide," as used herein, is meant a polypeptide that is substantially identical to, or is identical to, a mammalian kisspeptin polypeptide or a fragment thereof, e.g., a C-terminal fragment. Desirably, the kisspeptin polypeptide is a human kisspeptin polypeptide, for example, one comprising amino acids 68-121 or 112-121 of kisspeptin-1. In other desirable embodiments, a kisspeptin polypeptide may comprise amino acids 68-119 of the murine *KiSS-1* (GenBank Accession No. AF472576) gene product. Furthermore, the kisspeptin polypeptide desirably can alter a GPR54 biological activity.

By a "candidate compound" or "test compound" is meant a chemical, be it naturally-occurring or artificially-derived, that is surveyed for its ability to alter a biological activity of GPR54, e.g., in one of the assay methods described herein. Candidate or test compounds include, for example, peptides, polypeptides, synthetic organic molecules, naturally-occurring organic molecules, nucleic acid molecules, and

components thereof. Exemplary candidate compounds may be members of the RFamide (neuropeptides terminating in -Arg-Phe-NH_2) and RWamide families (Clements et al., Biochem. Biophys. Res. Commun. 284:1189-1198 (2001).

By "substantially identical" is meant a polypeptide or nucleic acid exhibiting at least 50%, preferably 60%, 70%, 75%, or 80%, more preferably 85%, 90% or 95%, and most preferably 99% identity to a reference amino acid or nucleic acid sequence. For polypeptides, the length of comparison sequences will generally be at least 10 amino acids, desirably at least 15 contiguous amino acids, more desirably at least 20, 25, 50, 75, 90, 100, 150, 200, 250, 300, or 350 contiguous amino acids, and most desirably the full-length amino acid sequence. For nucleic acids, the length of comparison sequences will generally be at least 45 contiguous nucleotides, desirably at least 60 contiguous nucleotides, more desirably at least 75, 150, 225, 275, 300, 450, 600, 750, 900, or 1000 contiguous nucleotides, and most desirably the full-length nucleotide sequence.

Sequence identity may be measured using sequence analysis software on the default setting (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Such software may match similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine, valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

Multiple sequences may also be aligned using the Clustal W(1.4) program (produced by Julie D. Thompson and Toby Gibson of the European Molecular Biology Laboratory, Germany and Desmond Higgins of European Bioinformatics Institute, Cambridge, UK) by setting the pairwise alignment mode to "slow," the pairwise alignment parameters to include an open gap penalty of 10.0 and an extend gap penalty of 0.1, as well as setting the similarity matrix to "blosum." In addition, the multiple alignment parameters may include an open gap penalty of 10.0, an extend gap penalty of 0.1, as well as setting the similarity matrix to "blosum," the delay divergent to 40%, and the gap distance to 8.

By a "functional fragment," as used herein, is meant an amino acid sequence that is substantially identical to a fragment, e.g., 5, 10, 15, 20, 15, 30, 50, 75, or 100 contiguous amino acids, of a polypeptide encoded by GenBank Accession No. AY029541, AF343725, NM_032551, or AY253981. In more desirable embodiments, a "functional fragment" is identical to 5, 10, 15, 20, 15, 30, 50, 75, or 100 contiguous amino acids of GenBank Accession No. AY029541, AF343725, NM_032551, or AY253981, or may be the entire amino acid sequence of the polypeptide encoded by GenBank Accession No. AY029541, AF343725, NM_032551, or AY253981. In addition, a "functional fragment" of a polypeptide has at least one biological activity of the full-length GPR54 polypeptide.

By "purified" is meant separated from other components that naturally accompany it. Typically, a factor is substantially pure when it is at least 50%, by weight, free from proteins, antibodies, and naturally-occurring organic molecules with which it is naturally associated. Preferably, the factor is at least 75%, more preferably, at least 90%, and most preferably, at least 99%, by weight, pure. A substantially pure factor may be obtained by chemical synthesis, separation of the factor from natural sources, or production of the factor in a recombinant host cell that does not naturally produce the factor. Proteins, vesicles, and organelles may be purified by one skilled in the art using standard techniques, such as those described by Ausubel et al. (*Current Protocols in Molecular Biology*, Wiley Interscience, New York, 2001). The factor is preferably at least 2, 5, or 10 times as pure as the starting material, as measured using polyacrylamide gel electrophoresis, column chromatography, optical density, HPLC analysis, or Western analysis (Ausubel et al., *Current Protocols in Molecular Biology*, Wiley Interscience, New York, 2001). Preferred methods of purification include immunoprecipitation, column chromatography such as immunoaffinity chromatography, magnetic bead immunoaffinity purification, and panning with a plate-bound antibody.

By "mutation" is meant an alteration in a naturally-occurring or reference nucleic acid sequence, such as an insertion, deletion, frameshift mutation, silent mutation, nonsense mutation, missense mutation, or one that affects splicing. In a desirable embodiment, a mutation is a single nucleotide polymorphism. In another

desirable embodiment, the amino acid sequence encoded by the nucleic acid sequence has at least one amino acid alteration relative to a reference sequence (e.g., a polypeptide encoded by GenBank Accession No. AY029541, AF343725, NM_032551, or AY253981). For example, a 443T>C alteration in the human GPR54 nucleic acid sequence results in a serine for leucine substitution at position 148 of corresponding amino acid sequence, the 991C>T alteration in the human GPR54 nucleic acid sequence results in the replacement of arginine at position 331 of the corresponding amino acid sequence with a premature stop codon, or the 1195T>A alteration in the human GPR54 nucleic acid sequence results in the replacement of the stop codon at residue 399 of the corresponding amino acid sequence with an arginine. A mutation in a GPR54 nucleic acid or amino acid sequence may be used to diagnose a patient as having or as being at risk of acquiring, for example, IHH, amenorrhea, microphallus, hypospadius, polycystic ovarian disease, prostate cancer, endometriosis, or uterine fibroids using standard methods in the art, such as those described herein.

Examples of recombinant DNA techniques for altering the genomic sequence of a cell, embryo, fetus, or mammal include inserting a DNA sequence from another organism (e.g., a human) into the genome, deleting one or more DNA sequences, and introducing one or more base mutations (e.g., site-directed or random mutations) into a target DNA sequence.

"Reproductive disorder" as used herein, refers to a malfunction or malformation of the reproductive system. Exemplary reproductive disorders include central precocious puberty, delayed puberty, prostate cancer, endometriosis, polycystic ovarian disease, uterine fibroids, IHH, amenorrhea, microphallus, and hypospadius.

"Central precocious puberty," as used herein, refers to the appearance of any primary or secondary indications of puberty in an individual at a time that is greater than two standard deviations before the onset of puberty in individuals of the same sex in a population.

"Delayed puberty," as used herein, refers to the absence of development of any signs or symptoms of sexual maturation, e.g., testicular enlargement, elevation of serum testosterone level, or a growth spurt in males and breast development,

menarche, or a growth spurt in females, at an age that is greater than two Standard Deviations away from what is normal for a given population or ethnic group, e.g., Caucasian, Hispanic, or African-American.

"Amenorrhea," as used herein, refers to the absence of the normal initiation of menses or to the cessation of menses for at least 3 months in a woman with previously normal menstrual cycles. Amenorrhea can be due to many etiologies including anatomic causes, primary ovarian failure, and chronic anovulation. One of the neuroendocrine profiles that can be seen in patients with anovulation is a hypogonadotropic state in which there is little to no stimulation of the pituitary gland by the hypothalamus (GnRH). This disorder is further described in: Martin et al., "Management of ovulatory disorders with pulsatile gonadotropin-releasing hormone," J. Clin. Endocrinol. Metab. 71:1081A-1081G, 1990; Perkins et al., "Neuroendocrine abnormalities in hypothalamic amenorrhea: spectrum, stability, and response to neurotransmitter modulation" J. Clin. Endocrinol. Metab. 84:1905-11, 1999; and Perkins et al., "Aetiology, previous menstrual function and patterns of neuro-endocrine disturbance as prognostic indicators in hypothalamic amenorrhoea," Hum. Reprod. 16:2198-205, 2001.

"Microphallus," as used herein, refers to a stretched penile length that is less than 2.5 cm at birth. Microphallus can be observed in states of severe androgen deficiency, and, therefore, can serve as an early physical examination marker of future hypogonadism. This disorder is further described in: Bin-Abbas et al., "Congenital hypogonadotropic hypogonadism and micropenis: effect of testosterone treatment on adult penile size; why sex reverasal is not indicated," J. Pediatr. 134:579-583, 1999; and Pitteloud et al., "The role of prior pubertal development, biochemical markers of testicular maturation, and genetics in elucidating the phenotypic heterogeneity of idiopathic hypogonadotropic hypogonadism," J. Clin. Endocrinol. Metab. 87:152-160, 2002.

"Hypospadius," as used herein, refers to the incomplete fusion of the penile urethra. Hypospadius is one of the most common congenital anomalies, having an

incidence of 4 to 8 per 1000 male births. This disorder is further described in: Carter, "Multifactorial genetic disease" In: McKusick And Claiborne, eds. Medical Genetics. NewYork: HP Publishing, 1973.

"Polycystic ovary disease," as used herein, refers to a clinical syndrome characterized by chronic oligoamenorrhea and hyperandrogenism, either evident on physical examination (i.e. acne or hirsutism) or with elevated levels of serum androgens in the bloodstream. This disorder affects 5-7% of all reproductive age women, making it the most common endocrinopathy in this group. The ovaries are studded with atretic follicles. Close to 50% of patients with polycystic ovary disease ("PCOS") are found to have frank diabetes, impaired glucose tolerance, or impaired insulin resistance at the time of diagnosis. In addition to abnormalities in the insulin/glucose axis, the neuroendocrine axis also appears to be abnormal in PCOS. This disorder is further described in: Kazer et al., "Circulating luteinizing hormone pulse frequency in women with polycystic ovary syndrome," J. Clin. Endocrinol. Metab. 65:233-236, 1987; Waldstreicher et al., "Hyperfunction of the hypothalamic-pituitary axis in women with polycystic ovarian disease: indirect evidence for partial gonadotroph desensitization," J. Clin. Endocrinolo. Metab. 66:165-172, 1988; and Dunaif, "Insulin resistance and the polycystic ovary syndrome: mechanism and implications for pathogenesis," Endocr. Rev. 18:774-800, 1997.

"Prostate cancer," as used herein, refers to a neoplasm of the prostate. This is the most common form of cancer in men, outside of non-melanoma skin cancer. Androgen deprivation via hormone therapy has traditionally been offered to men with locally advanced or metastic prostate cancer, although new combinations of therapies are currently being studied. Androgen ablation is accomplished via a GnRH agonist, which desensitizes the gonadotrophs, and ultimately results in loss of gonadotroph stimulation to the testicle. This disorder is further described in: Garnick, "Prostate cancer: screening, diagnosis, and management," Ann. Intern. Med. 118:804-818, 1993; Kramer et al., "Prostate cancer screening: what we know and what we need to know," Ann. Intern. Med. 119:914-923, 1993; and Wirth et al., "Bicalutamide

(Casodex) 150 mg as immediate therapy in patients with localized or locally advanced prostate cancer significantly reduces the risk of disease progression," Urology 58:146-51, 2001.

"Endometriosis," as used herein, refers to the presence of endometrial glands and stroma outside the endometrial cavity and uterine musculature. Endometriosis is found in 21-48 % of women undergoing laparoscopy for infertility. The most common symptoms of endometriosis are pelvic pain, dysmenorrhea, dyspareunia, abnormal menstrual bleeding, and infertility. This disorder is further described in: Conn and Crowley, "Gonadotropin-releasing hormone and its analogs," Annu. Rev. Med. 45:391-405, 1994; Sangi-Haghpeykar and Poindexter, "3rd Epidemiology of endometriosis among parous women" Obstet. Gynecol. 85:983-92, 1995; Dlugi et al., "depot (leuprolide acetate for depot suspension) in the treatment of endometriosis: a randomized, placebo-controlled, double-blind study. Lupron Study Group," Fertil. Steril. 54:419-27, 1990; and Ling, "Randomized controlled trial of depot leuprolide in patients with chronic pelvic pain and clinically suspected endometriosis. Pelvic Pain Study Group," Obstet. Gynecol. 93:51-58, 1999.

"Uterine fibroids," as used herein, refer to benign leiomyomas or myomas arising from the smooth muscle cells of the uterus. Uterine fibroids are present in 25% of reproductive aged women. Genetics, hormones, and growth factors all play a role in the formation and growth of these tumors. The most common symptoms are excessive bleeding, pain, and infertility. This disorder is further described in: Buttram and Reiter, "Uterine leiomyomata: etiology, symptomatology, and management," Fertil. Steril. 36:433-434, 1981; Friedman et al., "A randomized, double-blind trial of a gonadotropin releasing-hormone agonist (leuprolide) with or without medroxyprogesterone acetate in the treatment of leiomyomata uteri," Fertil. Steril. 49:404-409, 1988; Carr et al., "An evaluation of the effect of gonadotropin-releasing hormone analogs and medroxyprogesterone acetate on uterine leiomyomata volume by magnetic resonance imaging: a prospective, randomized, double blind, placebo-controlled, crossover trial," J. Clin. Endocrinol. Metab. 76:1217-23, 1993; Conn and Crowley, "Gonadotropin-releasing hormone and its analogs," Annu. Rev. Med. 45:391-405, 1994; and Stewart, "Uterine fibroids," Lancet 357:293-298, 2001.

The present invention provides a number of advantages. For example, current treatments for central precocious puberty, prostate cancer, endometriosis, uterine fibroids, preparation for *in vitro* fertilization, and contraception generally use GnRH or GnRH analogs to produce a state of hypogonadotropic hypogonadism for therapeutic purposes. However, most currently available GnRH analogues are difficult to administer, especially orally. In contrast, the claimed methods can be used to identify small molecule regulators of GPR54 which may be administered orally as well as parentally to produce a similarly hypogonadotropic state and would thereby simplify the treatment of such reproductive conditions. Moreover, these methods facilitate the identification of novel contraceptive compounds.

Other features and advantages of the invention will be apparent from the following Detailed Description, the drawings, and the claims.

Brief Description of the Drawings

FIGURE 1A is a series of charts showing the sequence analysis of *GPR54* from an affected individual from the index pedigree (WT; left-hand column) and the patient with the compound heterozygous mutations (right-hand column); 443T>C in exon 3 substitutes a serine for the normal leucine (L148S); 991C>T in exon 5 replaces an arginine with a premature stop codon (R331X); and 1195T>A in exon 5 replaces the stop codon at residue 399 with an arginine (X399R).

FIGURE 1B is a sequence alignment showing the evolutionary conservation of *GPR54*. The galanin and somatostatin receptors have the closest homologies to the receptor encoded by *GPR54* by BLAST analysis (SEQ ID NOS:35-45). The consensus 7 transmembrane sequence (SEQ ID NO:46) was derived from Washington University (St. Louis, Missouri) database of protein family alignments (www.pfam.wustl.edu).

FIGURE 1C is a scanned image of a gel showing the RT PCR results from lymphoblasts taken from a patient bearing R331X and X399R mutations. Lane 1 is the size marker; lanes 2-6 show RT and PCR amplification results from an arbitrarily chosen gene, *PAK6*. These reactions were performed simultaneously and under identical conditions with *GPR54* to serve as internal controls. The amplification

primers for *PAK6* were placed in exons 6 and 9 to cross splice junctions. In particular, the samples are as follows: Lane 2: RNA from patient with R331X /X399R; Lane 3: Control RNA (normal volunteer); Lane 4: Control RNA: -RT; Lane 5: Control genomic DNA; Lane 6: Distilled H_20; Lane 7: Size marker; Lanes 8-12: RT PCR of *GPR54*. Primer set #1 placed pre- and post polyA site, fragment size = 459 base pairs; Lane 8: RNA from patient with R331X /X399R; Lane 9: Control RNA (normal volunteer); Lane 10: Control RNA: -RT; Lane 11: Control genomic DNA; Lane 12: Distilled H_20; and Lane 13: Size marker.

FIGURE 2 is a schematic diagram of the GPR54 polypeptide (No shading = exon 1; light gray shading = exon 2; right diagonals = exon 3; dark gray shading = exon 4; left diagonals = exon 5). The positions of the transmembrane domains were predicted by the TMPred program for the prediction of membrane-spanning regions and their orientation (http:/www.ch.embnet.org/software/TMPRED_form.html). The mutated amino acids are enlarged.

FIGURES 3A-3C are a series of charts showing biochemical phenotyping in a patient carrying the *GPR54* mutations R331X and X399R. The shaded area for LH in Figures 3A and 3B represents the mean ± 2SD as determined in 20 normal men. Figure 3A is the baseline secretory pattern of LH with gonadotropin determinations made at 10 min intervals for 24 h. Figure 3B shows the LH response to exogenous pulsatile GnRH administered IV over a range of doses. The dose order was randomized. The patient received exogenous pulsatile GnRH SC q for 2 hours as an outpatient for 11 months prior to study. Figure 3C shows the dose response curve of a patient drawn with a regression line. The patient's data fall to the left of the 95% confidence limits defined by the mean LH amplitude of 6 IHH men (no coding sequence abnormalities in *GPR54*) undergoing the same protocol.

FIGURE 4 is a flow-chart showing an exemplary conceptual framework for understanding defects that cause hypogonadotropic hypogonadism in the absence or presence of anosmia.

FIGURES 5A-5D are a series of dose response curves of ligand stimulated inositol phosphate production for mutant constructs, corrected for protein content. The data are expressed as mean ± SEM of multiple replicates, each done in triplicate.

Co-transfection of beta galactosidase revealed no differences in transfection efficiencies. (Quantitative RT PCR and Western blotting revealed equivalent transcript and protein expression). Figure 5A shows a comparison between wild-type and L148S constructs (n=3 independent experiments, each done in triplicate). Figure 5B shows a comparison between wild-type and R331X constructs (n=2 independent experiments, each done in triplicate). Figure 5C shows a comparison between wild-type and X399R polyA stop constructs (n=2 independent experiments, each done in triplicate). Figure 5D shows relative quantification of wild-type to mutant *GPR54* allele expression in lymphoblastoid cell lines using quantitative RT PCR.

FIGURE 6A is a schematic diagram showing a targeted deletion allele of *Gpr54*. Exons are shown in open boxes and resistance cassettes and markers are shown in filled boxes. The locations of several restriction sites, primers and probes are shown above the loci.

FIGURE 6B is a Southern blot demonstrating correct targeting of 5' and 3' arms. The mutant and wild-type bands detected using probes after diagnostic restriction digests are indicated with arrows. The fragment sizes are in base pairs.

FIGURE 6C is an image of a blot showing that homozygous mutant GPR54 mice show ablated transcription 3' to the locus deletion. RT PCR analysis spanning exons 4 and 5 shows the absence of a detectable transcript in the 3' end of the locus homozygous for GPR54 deletion. (Lane 1= wild-type, Lane 2= heterozygote, Lane 3= homozygote, Lane 4= genomic DNA control, and Lane 5= RT- control.

FIGURES 7A-7N are a series of images showing the gonadal anatomy and secondary sexual characteristics of *Gpr54* -/- mice. Figure 7A shows a reduction in testes size (wild type and mutant males) and Figure 7B shows small ovaries and uteri in *Gpr54* -/- females. Scale bar = 0.5 cm. A comparison of Figures 7C and 7D shows a reduction in number of spermatozoa in seminiferous tubules of mutant mice (Fig. 7C = *Gpr54* +/+, Fig. 7D = *Gpr54* -/-). Scale bar = 50 μm. A comparison of Figure 7E and 7F shows an absence of sperm in the mutant epididymis (Fig. 7E = *Gpr54* +/+, Fig. 7F = *Gpr54* -/-). Scale bar = 100 μm. A comparison of Figures 7G and 7H shows reduced preputial gland development in mutant mice (Fig. 7G = *Gpr54* +/+, Fig. 7H = *Gpr54* -/-). Scale bar = 1 cm. A comparison of Figures 7I and 7J shows

retention of prepubescent zone X in the adrenal gland of mutant mice (Fig. 7I = *Gpr54* +/+, Fig. 7J = *Gpr54* -/-). Scale bar = 20 μm. A comparison of Figures 7K and 7L shows reduced mammary duct formation in mutant mice (Fig. 7K = *Gpr54* +/+, Fig. 7L = *Gpr54* -/-). The dark mass is a lymph node. Scale bar = 0.5 cm. A comparison of Figures 7M and 7N shows the absence of Graffian follicles and corpora lutea in mutant mice (Fig. 7M = *Gpr54* +/+, Fig. 7N = *Gpr54* -/-)(CL = corpus luteum). Scale bar = 300 μm.

FIGURES 8A-8G are a series of graphs showing hormone levels in *Gpr54* -/- mice. 5-8 animals were used in each assay group. Figure 8A shows testosterone levels, Figure 8B shows 17β estradiol levels, Figure 8C shows follicle stimulating hormone (FSH) levels, Figure 8D shows luteinizing hormone levels, Figure 8E shows gonadotropin releasing hormone levels in the hypothalamus, Figure 8F shows luteinizing hormone release after gonadotropin releasing hormone injection, and Figure 8G shows a change in pituitary luteinizing hormone concentration after gonadotropin releasing hormone injection. Statistically significant differences are indicated (Non-parametric Mann-Whitney tests apart from panel A which used an unpaired Student t test).

FIGURE 9A is a graph showing the effect of central administration of vehicle (n=4), 30 μg metastin (n=3), 100 μg metastin (n=4) or 100 μg metastin with acyline pretreatment (n=3) on GnRH release in juvenile orchidectomized rhesus monkeys, as reflected by plasma LH levels (mean±SEM).

FIGURE 9B is a graph showing the comparison of mean±SEM LH concentrations following central vehicle or metastin injections in juvenile orchidectomized rhesus monkeys (n=3-4). Bars with different letters differ (P 0.05).

FIGURE 10 is a graph showing the effects of kisspeptin-10 and kisspeptin–54 (1 nmol, delivered ICV) on serum levels of LH in a mouse, measured at 30 min following a bolus injection (* $p < 0.001$ vs. vehicle alone).

FIGURE 11 is a graph showing the effects of different doses of kisspeptin-54 (metastin) (ranging from 0 to 5 nmol delivered ICV) on serum levels of LH in a mouse, measured 30 min following a bolus injection (* $p < 0.01$ vs. vehicle; ** $p < 0.001$ vs. vehicle; + $p < 0.001$ vs. 0.001 pmol metastin).

FIGURES 12A and 12B are a series of graphs showing the effects of metastin (50 pmol delivered ICV) or its vehicle alone in a mouse, coupled with pretreatment with either a GnRH antagonist, acyline (50 μg, sc) or its vehicle alone. Figure 12A depicts LH data and Figure 12B depicts FSH data (S/V = saline/vehicle, S/M = saline/metastin, A/V = acyline/vehicle, A/M = acyline/metastin; * $p < 0.001$ S/M vs. all other treatments).

FIGURES 13A-13F are a series of images showing the distribution of *KiSS-1* mRNA-expressing cells in the hypothalamus of a mouse. *KiSS-1* mRNA-expressing cells are indicated by clusters of white dots, corresponding to clusters of silver grain where the labeled RNA probe has been concentrated. *KiSS-1* mRNA-containing cells were observed in the anteroventral periventricular nucleus (Figure 13A), the periventricular nucleus (Figure 13B), anterodorsal preoptic nucleus (Figure 13C), the medial amygdala (Figure 13D), and the arcuate nucleus (Figures 13E and 13F) (3V, Third ventricle; AC, anterior commissure; OT, optic tract; OX, optic chiasm).

Detailed Description

Identifying the signals which modulate pulsatile GnRH secretion or a cell's response to GnRH can be used to understand and design therapeutic approaches (both stimulatory and suppressive) for the numerous human disorders that might benefit from either increasing or decreasing GnRH secretion, including idiopathic hypogonadotropic hypogonadism, amenorrhea, microphallus, microspadia, polycystic ovarian disease, endometriosis, uterine fibroids, and prostate cancer, as well as treatments for infertility and contraception (Conn and Crowley, "Gonadotropin-releasing hormone and its analogues," N. Engl. J. Med. 324:93-103, 1991).

The genetic and molecular basis for idiopathic hypogonadotropic hypogonadism (IHH) without anosmia, a condition characterized by the failure of the anterior pituitary to produce gonadotropins, was previously largely unknown save defects in the GnRH receptor. Most patients carrying the diagnosis of IHH respond to exogenous administration of gonadotropin hormone releasing hormone (GnRH), a peptide normally produced by the hypothalamus. Therefore, the pathophysiologic defect of IHH has been assumed to be hypothalamic in origin. However, due to the

infertility caused by untreated gonadotropin deficiency and the resultant small families, genetic approaches aimed at identifying the genes involved in IHH had been difficult to employ in this disease model.

We identified a large, consanguineous Saudi family in which normosmic IHH was inherited as an autosomal recessive trait. Although the total number of affected individuals within the pedigree was modest (n=6), 3 consanguineous marriage loops provided substantial statistical power. Genome-wide linkage analysis defined a new disease locus on chromosome 19p13.3. Screening within this candidate region implicated a 7 transmembrane domain G protein-coupled receptor, *GPR54*. Mutation analysis, *in vitro* studies, and *in vivo* dose responsiveness to exogenous GnRH establish mutations in this gene as a cause of IHH.

Exemplary mechanisms by which abnormalities in GPR54 may cause IHH are shown in Figure 4. Moreover, abnormalities in GnRH secretion or an individual's response to GnRH can lead to a variety of other disorders, including amenorrhea, microphallus, microspadia, and polycystic ovarian disease. In addition, induction of hypogonadotropic hypogonadism by suppression of endogenous GnRH secretion, is a valuable therapy for central precocious puberty, endometriosis, uterine fibroids, prostate cancer, contraception, and as a preparation for in vitro fertilization.

The present invention features the use of hypothalamic receptor, GPR54, which is mutated in humans and as a result causes an autosomal recessive form of IHH. The identification of this hypothalamic receptor as a key modulator of normal GnRH secretion not only enables its use in identifying compounds that may be used to treat IHH, but also compounds that may be used to decrease GnRH secretion and produce hypogonadotropic hypogonadism that has already been proven useful as a treatment for several reproductive disorders such as central precocious puberty, prostate cancer, endometriosis, polycystic ovarian disease, uterine fibroids, as a potential contraceptive agent, and as a preparation for in vitro fertilization.

The following examples are provided for the purpose of illustrating the invention and should not be construed as limiting.

Example 1

Association of GPR54 with Reproductive Disorders

A genome-wide scan of 21 individuals, including the 6 individuals affected by idiopathic hypogonadotropic hypogonadism, from a consanguineous Saudi family was performed. Manual haplotypes were constructed and additional fine mapping was performed using commercially available markers, markers designed in-house, and single nucleotide polymorphisms. A shared haplotype spanning a 1.06 Mb interval was identified (Acierno et al., "A locus for autosomal recessive idiopathic hypogonadotropic hypogonadism on chromosome 19p13.3," J. Clin. Endocrinol. Metab. 88(6):2947-2950, 2003). A search of the Human Genome Browser (November 2002 Assembly) revealed 23 known genes including *GPR54*, a G protein-coupled receptor expressed in the human brain, pituitary, and placenta (Muir et al., "AXOR12, a novel human G protein-coupled receptor, activated by the peptide Kiss-1," J. Biol. Chem. 276:28969-28975, 2001; Kotani et al., "The metastasis suppressor gene Kiss-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54," J. Biol. Chem. 276:34631-34636, 2001). *GPR54* has 5 exons and contains an open reading frame of 1197 base pairs that encodes a 398 amino acid protein.

When the coding sequence of *GPR54* was examined for DNA sequence variants, a homozygous single nucleotide variant (443T>C) in exon 3, which substitutes a serine for the normal leucine at position 148, was discovered in all affected individuals and segregated properly within the family according to carrier status (Figure 1; L148S; all references to base pair positions reported according to standard numbering and nomenclature (Antonarakis, "Nomenclature Working Group. Recommendations for a nomenclature system for human gene mutations," Hum. Mut. 11:1-3, 1998). The variant does not appear to be a simple polymorphism as it: (1) was found only in affected family members but not in unaffected relatives with normal puberty and reproduction; (2) was absent in 160 control chromosomes from unrelated, unaffected North American control subjects and 100 chromosomes from Middle Eastern controls; (3) is present in an amino acid residue that is conserved across species including mouse, rat, amphioxus, and pufferfish (GenBank Accession

#AF343726, #BAB55447, #AAM18884, and Fugu Genome Server accession SINFRUP00000071513 (Aparicio et al., Science 297:1283-1285, 2002), respectively (see also, Figure 1); and (4) alters the polarity of the encoded amino acid from hydrophobic to neutral and therefore, might be expected to alter receptor function.

To determine whether changes in *GPR54* could be found in other patients with IHH, DNA samples were sequenced from 63 unrelated individuals with normosmic IHH and 20 patients with KS (Kallmann synsdrome). In one African-American male, a C to T transition was identified at nucleotide 991 in exon 5, replacing an arginine at residue 331 with a premature stop codon (991C>T (R331X)). Additionally, a T to A transversion was identified at nucleotide 1195 in exon 5, replacing the stop codon at residue 399 with an arginine (1195T>A (X399R)). This removal of a stop codon results in the continuation of the open reading frame into the polyA tail. The X399R change is predicted to result in at least 247 additional nucleotides, potentially resulting in a mutant receptor with at least an additional 83 amino acids added to its C-terminal tail (Figure 1). Neither change was identified in 160 North American control chromosomes or 100 African-American control chromosomes. A schematic of all mutations is depicted in Figure 2.

To confirm that the nonsense and nonstop mutations exist on separate chromosomes, allele-specific cloning of an amplified genomic fragment containing both changes was performed. Of the resulting 17 clones containing inserts, nine clones were found to contain only the R331X change; 8 clones were found to contain only the X399R change; and no clones were found to contain both R331X and X399R, confirming that the two variants exist on separate alleles (compound heterozygote).

RT PCR

RT PCR products generated from the Saudi pro-band and the additional 10 IHH patients were of the expected size for all segments, and sequence analysis of these products revealed that the newly-identified mutations did not result in cryptic splicing. To address whether the nonstop mutation X399R extended transcript length, primers were designed to amplify specific segments within the 3'-UTR. The polyA

signal begins at nucleotide 1391 (194 base pairs after the physiologic stop codon), and the polyA tail begins at position 1442 (51 base pairs downstream from the initiation of the polyA signal). RT-PCR from the patient bearing the compound heterozygous mutations (R331X, X399R) revealed no evidence for a transcript containing sequence corresponding to the region 3' to the polyA sequence. Therefore, primer set #1, designed to amplify a 459 base pair fragment incorporating extending beyond the polyA site failed to produce a band from the patient's RNA (Figure 1C). Of note, an appropriate sized band was detected from the patient's genomic DNA (demonstrating the integrity of the primers) and amplification of the patient's cDNA was successfully performed on a "control" gene, (demonstrating a successful reverse transcriptase reaction and absence of genomic contamination). Similarly, primer set #2, amplifying a 174 base pair fragment downstream from the polyA signal, failed to amplify a band from the patient's RNA.

The nature of the discovered changes in *GPR54* and the absence of these changes in a control population provided strong evidence that mutations in *GPR54* cause IHH. Accordingly, we also refer to the protein product of the *GPR54* gene as hypogonadotropin-1.

IP Accumulation in Response to Kisspeptin 112-121

The *GPR54* gene product has been previously shown to increase inositol phosphate (IP) production in response to peptides derived from kisspeptin-1 (Kotani et al., "The metastasis suppressor gene Kiss-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54," J. Biol. Chem. 276:34631-34636, 2001). To determine if the identified changes in GPR54 affect receptor function, IP production was measured in transiently transfected COS-7 cells in response to kisspeptin-1 112-121. In the L148S mutant, the maximal percent inositol phosphate response was decreased 65 percent compared to wild-type (Figure 5A). In the R331X mutant, the maximal percent inositol phosphate response was decreased 67 percent compared to wild-type (Figure 5B). RT PCR from COS-7 cells transfected with the X399R construct revealed a transcript that contained 3'UTR, polyA tail, as well as expression vector sequence. In the absence of the physiological stop codon at position

399 (but a stop codon in the vector sequence), the *in vitro* transcript resulted in an elongated receptor protein. Because this in vitro construct did not accurately mimic in vivo physiology, it was not used in the functional studies. The X399R polyA stop construct, which makes a protein identical to that encoded by the nonstop transcript, stimulates inositol phosphate production 61 percent of wild type (Figure 5C). No fold increment for IP stimulation was observed for pCMVsport6 (negative control).

Quantitative RT-PCR

Expression analysis of the *GPR54* alleles was performed utilizing Taqman real-time PCR with lymphoblast mRNA as a template. The levels of the mutant alleles were expressed relative to *GPR54* gene expression from control lymphoblasts. When compared to the standardized control mRNA, the total *GPR54* mRNA concentration from the compound heterozygote patient was 17 ± 2 percent (mean ± SEM) (*t* test, $P<0.001$); the R331X allele was 17 ± 2 percent while the expression level of the X399R was 3 ± 0.3 percent of normal (Figure 5D).

Endocrinological Phenotyping

A baseline luteinizing hormone (LH) profile from the pro-band containing the heterozygous mutations R331X and X399R is depicted in Figure 3A. On q 10 min blood sampling, low LH levels in the setting of low testosterone are evident, particularly as compared to the mean LH level (± 2SD) as determined in 20 normal men sampled at 10 min intervals for 24 h as previously reported (Spratt et al., Am. J. Physiol. 254:E658-E666, 1988; and Whitcomb and Crowley, J. Clin. Endocrinol. Metab. 70:3-7, 1990). Nonetheless, several low-amplitude luteinizing hormone pulses (n=9) are present as determined by formal pulse analysis. The response to 4 doses of GnRH IV are graphed in Figure 3B and compared to the mean LH amplitude of 6 other IHH patients treated with the identical regimen (Figure 3C). The pro-band with mutations in *GPR54* demonstrates a left shifted dose response curve that lies outside the 95% confidence limits of the mean data of men with IHH lacking any GPR54 mutations, suggesting that the patient with R331X and X399R is more sensitive to GnRH than other IHH patients.

Pathophysiology of Homozygous *Gpr54* Deficient Mice: Anatomy and Behavior

Gpr 54-deficient mice have striking physiological similarities to patients with IHH, including a lack of sexual maturation associated with low levels of gonadotrophins. In addition, their gonads remained sensitive to exogenous gonadotropins and their pituitary gonadotropes remained responsive to gonadotropin releasing hormone stimulation. This strong fidelity of the human and mouse models establishes a central role for GPR54 in gonadotropin releasing hormone secretion and the onset of sexual maturation across mammalian species. Moreover, the *Gpr54*-deficient mouse permitted quantitation of hypothalamic gonadotropin releasing hormone levels that were normal in the face of their hypogonadotropism. The presence of normal levels of gonadotropin releasing hormone in the hypothalamus of *Gpr54*-deficient, sexually immature mice is reminiscent of the prepubertal rat and monkey who have normal numbers of gonadotropin releasing hormone-containing neurons, mRNA levels, and gonadotropin releasing hormone in the hypothalamus (Wiemann et al., Endocrinology 124:1760-1767, 1989; and Cameron et al., Biol. Reprod. 33:147-156, 1985).

Homozygous mutant mice (*Gpr54* -/-) (Figures 6A and 6B) were viable and obtained at the expected Mendelian frequency from heterozygous breeding pairs. RT PCR analysis of transcripts showed no detectable transcription in the 3' end of the homozygous *Gpr54* ^{tm1PTL} allele (Figure 6C). *Gpr54* +/- mice were phenotypically normal and fertile. *Gpr54* -/- mice did not display any of the physiologic changes associated with sexual maturation. *Gpr54* -/- testes were significantly smaller than age matched controls (Figure 7A) (0.05 ± 0.005 g, n=9, vs. 0.18 ± 0.01 g, n=8, $P<0.0001$, Mann-Whitney U-statistic) and did not contain spermatozoa in the lumen of the seminiferous tubules or the epididymis (Figures 7C-7F). Primary spermatocytes were present but very few haploid spermatids were present suggesting that spermatogenesis had been initiated but stopped before the meiotic division stage. Male mice also showed lack of secondary sex gland development of the preputial gland (Figures 7G and 7H), the seminal vesicles (not shown) and the prostate (not shown). In the adrenal glands of the mutant animals, the innermost region of the cortex which normally regresses at puberty (zone X) was still present (Figures 7I and

7J). Sexual mounting behavior was also not observed in the males. No gross morphological abnormalities in the central nervous system of *Gpr54* -/- mice were found and mutants thrived, apart from the reproductive defect.

Female mutant mice also showed defective sexual development with small vaginal openings and failure to become pregnant after appropriate mating exposure. Vaginal smears consisted of non-keratinized epithelia and mucus strands similar to that observed in the immature females indicating lack of an estrus cycle (not shown). The uterine horns from *Gpr54* -/- mice were threadlike and the ovaries significantly smaller than normal (wild-type 5.7 ± 0.7 mg, n=9; mutant 1.0 ± 0.1 mg, n=8, P<0.001, unpaired Mann-Whitney U-statistic) (Figure 7B). Mammary tissue showed no post-pubertal maturation of branched epithelial ducts (Figures 7K and 7L). The ovaries contained primary and secondary follicles and occasionally an early antral follicle but no large Graaffian follicles or corpora lutea (Figures 7M and 7N).

Gpr54 Deficient Mice: Endocrine Phenotype

Gpr54 -/- male mice had significantly reduced blood testosterone levels compared to age-matched +/+ controls (mutants: 0.1 ± 0.02 pg/ml, n=12; wild type 4.6 ± 1.6 pg/ml, n=11, P<0.001 unpaired Mann-Whitney U-statistic). The testosterone levels of *Gpr54* -/- male mice was similar to those observed in *Gpr54* +/+ females (0.2 ± 0.02 pg/ml, n=8) (Figure 8A). The 17β estradiol levels of *Gpr54* -/- female mice were comparable to those of *Gpr54* +/+ females at non-estrus stages of the reproductive cycle (Figure 8B) and to the basal level of male *Gpr54* +/+ serum estradiol (data not shown). No *Gpr54* -/- females were identified that had a 17β estradiol level similar to that found at estrus (96.5 ± 16.3 pg/ml, n=5) (Figure 8B).

The lack of an estrus cycle in females was not caused by an inability of gonadal tissue to respond to gonadotropins. *Gpr54* -/- females could be induced to ovulate following sequential injection of the gonadotropins Pregnant Mares Serum (PMS) and human chorionic gonadotropin (hCG) (data not shown). The lack of an estrus cycle and failure to produce sperm in *Gpr54* -/- animals was caused by a significant reduction in serum FSH (P<0.01) and a more modest decrease in LH (Figures 8C and 8D). Possibilities for the reduced levels of circulating gonadotropins

include an absence of pituitary gonadotropes, an inability of existing gonadotropes to respond to gonadotropin releasing hormone stimulation, and a lack of gonadotropin releasing hormone production. The latter was excluded as measurement of gonadotropin releasing hormone protein in hypothalamic extracts showed no significant difference between normal and mutant animals (Figure 8E). In addition, measurement of pituitary luteinizing hormone and follicle stimulating hormone showed that while the total amount of each hormone was lower in *Gpr54* -/- than wild-type mice, significant quantities of each hormone were found, indicating that the pituitary gonadotropes are present in *Gpr54* -/- mice and are capable of synthesizing LH and FSH. Furthermore, adult *Gpr54* -/- female mice responded to injection of gonadotropin releasing hormone by secretion of LH into the blood stream (Figure 8F) with a corresponding depletion in pituitary LH content (Figure 8G). Similar results were obtained in response to gonadotropin releasing hormone injection for FSH secretion (data not shown). Although the absolute level of serum LH following gonadotropin releasing hormone injection was lower in *Gpr54* -/- mice than in +/+ animals, the proportionality of LH increase was similar (5X over basal). These responses are consistent with a "first exposure" to gonadotropin releasing hormone (McDowell et al., J. Endocrinol. 95:321-330, 1982; and McDowell et al., J. Endocrinol. 95:331-340, 1982).

Ligands for GPR54

GPR54 is a member of the rhodopsin family of G protein-coupled receptors with sequence homologies to members of the galanin receptor family, having 28, 30, and 30% amino acid identity with human GalR1, GalR2, and GalR3 respectively (Muir et al., "AXOR12, a novel human G protein-coupled receptor, activated by the peptide Kiss-1," J. Biol. Chem. 276:28969-28975, 2001). Although galanin does not bind to GPR54, (Muir et al., "AXOR12, a novel human G protein-coupled receptor, activated by the peptide Kiss-1," J. Biol. Chem. 276:28969-28975, 2001; Lee et al., "Discovery of a receptor related to galanin receptors," FEBS 446:103-107, 1999), endogenous ligands for this receptor have been isolated. These natural agonists, with a common theme of a RF-amide C terminus, derive from a precursor protein,

kisspeptin-1 (Ohtaki et al., "Metasis suppressor gene Kiss-1 encodes peptide ligand of a G-protein-coupled receptor," Nature 411:613-617, 2001; Muir et al., "AXOR12, a novel human G protein-coupled receptor, activated by the peptide Kiss-1," J. Biol. Chem. 276:28969-28975, 2001; Kotani et al., "The metastasis suppressor gene Kiss-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54," J. Biol. Chem. 276:34631-34636, 2001). Kisspeptin-1 was originally isolated by differential display and subtractive hybridization from melanoma cells suppressed for their metastatic potential after microcell-mediated transfer of human chromosome 6 (Miele et al., "Metastasis suppressed, but tumorigenicity and local invasiveness unaffected, in the human melanoma cell line MelJuSo after introduction of human chromosomes 1 or 6," Mol. Carcinog. 15:284-299, 1996; Lee et al., "KiSS-1, a novel human malignant melanoma metastasis-suppressor gene," J. Natl. Cancer Inst. 88:1731-1737, 1996; Lee and Welch, "Identification of highly expressed genes in metastasis-suppressed chromosome 6/human malignant melanoma hybrid cells using subtractive hybridization and differential display," Int. J. Cancer 71:1035-1044, 1997), a chromosome preferentially lost during the progression of many melanomas (Trent et al., "Identification of a recurring translocation site involving chromosome 6 in human malignant melanoma," Cancer Res. 49:420-423, 1989; Walker et al., "Simple tandem repeat allelic deletions confirm the preferential loss of distal chromosome 6q in melanoma," Int. J. Cancer 58:203-206, 1994; Takata et al., "Clonal heterogeneity in sporadic melanomas as revealed by loss-of-heterozygosity analysis," Int. J. Cancer 85:492-497, 2000). When searching for natural agonists of the then "orphan" nuclear receptor GPR54, placental tissue extracts were discovered by multiple groups to demonstrate biological activity; when the active peptides were purified by mass spectroscopy, they were found to be derived from kisspeptin-1 (Ohtaki et al., "Metasis suppressor gene Kiss-1 encodes peptide ligand of a G-protein-coupled receptor," Nature 411:613-617, 2001; Kotani et al., "The metastasis suppressor gene Kiss-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54," J. Biol. Chem. 276:34631-34636, 2001). The longest peptide (kisspeptin-1 68-121) is known as "mestastin" as predicted to result from proteolytic processing of the 121 amino acid parent protein. Metastin has recently been shown to be secreted

from the placenta throughout gestation (Horikoshi et al., "Dramatic elevation of plasma metastin concentration in human pregnancy: metastin as a novel placenta-derived hormone in humans," J. Clin. Endocrinol. Metab. 88:914-919, 2003). Shorter C-terminal peptides (including kisspeptin-1 112-121) share similar affinities and efficacies, and demonstrate that the C-terminal part of the peptide is responsible for the activation of GPR54 (Ohtaki et al., "Metasis suppressor gene Kiss-1 encodes peptide ligand of a G-protein-coupled receptor," Nature 411:613-617, 2001; Muir et al., "AXOR12, a novel human G protein-coupled receptor, activated by the peptide Kiss-1," J. Biol. Chem. 276:28969-28975, 2001; Kotani et al., "The metastasis suppressor gene Kiss-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54," J. Biol. Chem. 276:34631-34636, 2001).

In addition, ligands for GPR54 may include members of the RFamide (neuropeptides terminating in -Arg-Phe-NH_2) and RWamide families (Clements et al., Biochem. Biophys. Res. Commun. 284:1189-1198 (2001).

Kisspeptin regulates gonadotrophin secretion in the mouse

To determine whether metastin and kisspeptin-10 could stimulate LH release in adult male mice, metastin or kisspeptin-10 was administered via an ICV injection. In particular, the mice were handled daily for 2 weeks prior to the experiment. The mice were given an ICV injection of metastin (1 nmol) suspended in aCSF, kisspeptin-10 (1 nmol) suspended in artificial cerebrospinal fluid (aCSF) + 15% DMSO, aCSF + 15% DMSO alone or aCSF alone (n = 5 per group). Blood was obtained via orbital bleed 30 min post-injection and sera were assayed for LH. These experiments showed that metastin and kisspeptin-10 stimulated LH secretion in the mouse ($p = 0.01$ vs. vehicle treated animals) (Fig. 10). LH was not different in those injected with aCSF or aCSF + 15% DMSO (data not shown).

To determine the lowest effective dose of metastin to stimulate LH secretion, metastin was given at varying doses. The mice were given an ICV injection of metastin in doses varying from 1 fmol to 5 nmol or aCSF alone (n = 5-8 per group). Doses administered include 1 fmol, 10 fmol, 0.1 pmol, 1 pmol, 10 pmol, 0.1 nmol, 0.375 nmol, 0.625 nmol, 1.25 nmol, 2.5 nmol, and 5nmol, and blood was obtained via

orbital bleed 30 min post-injection and sera were assayed for LH. Clearly, metastin produced a significant increase in serum LH at all doses tested (Fig. 11). The responses to doses greater than 1 fmol were not significantly different from each other. The response to 1 fmol was intermediate-significantly greater than vehicle ($p<0.05$) but less than the 10 fmol and higher doses ($p<0.001$).

To determine whether metastin's stimulatory effect on gonadotropin secretion was mediated by GnRH, we pretreated animals with acyline, a potent GnRH antagonist, before delivering the ICV injection of metastin. Mice received a subcutaneous injection of acyline (50 µg) dissolved in sterile saline (100 µl/mouse) 24 hours and 1 hour prior to ICV injection of metastin or aCSF alone. Control mice were given a subcutaneous injection of saline only at 24 hours and 1 hour prior to ICV injections. On the day of the experiment mice received either an ICV injection of metastin (0.05 nmol) or aCSF alone. Of the mice that had been given acyline, half were treated with metastin and the other half received aCSF (n = 6/group). Similarly, half of the animals that had been treated with saline were given metastin and the other half received aCSF (n = 6). Blood was obtained by orbital bleed 60 min post-ICV injection and sera were assayed for LH and FSH.

From these experiments, it is clear that metastin (0.05 nmol) significantly stimulated both LH ($p<0.0001$) and FSH secretion ($p<0.001$) compared to vehicle-treated animals. Gonadotropin responses to metastin were blocked in mice pretreated with acyline (metastin vs vehicle: $p>0.05$) (Fig. 12). Data from the acyline/vehicle treated group are not shown.

The distribution of *KiSS-1* mRNA in the hypothalamus of the mouse was also determined. Silver grain clusters, representing cells expressing KiSS-1 mRNA, were found at several levels through the rostral-caudal extent of the hypothalamus. Many cells expressing KiSS-1 mRNA were observed in the anteroventral periventricular nucleus (AVPV), the periventricular nucleus (PeN), and the arcuate nucleus (ARC) (Fig. 13). In the rostral aspect of the arcuate nucleus, KiSS-1 mRNA was found throughout both the medial and lateral divisions. However, in the caudal aspect of the arcuate nucleus, KiSS-1 mRNA expression was restricted to the ventral portion of the nucleus. Some cells expressing KiSS-1 mRNA were observed in the anterodorsal

preoptic nucleus (ADP), whereas few cells were found in the medial amygdala and bed nucleus of the stria terminalis. (Muir and colleagues also report finding significant expression of KiSS-1 in several other areas of the forebrain in the human, such as the caudate nucleus, globus pallidus, nucleus accumbens, putamen, and striatum (Muir et al., J. Biol. Chem 276:28969-28975, 2001).) The amount of KiSS-1 mRNA per cell, as estimated by the number of silver grains per cluster, did not appear to differ significantly among these anatomical regions of the hypothalamus. Including excess unlabeled antisense probe with radiolabeled antisense probe abolished all specific signal, and no signal was observed following the application of radiolabeled sense probe.

All data above are expressed as a mean ± SEM for each group. Differences among groups were assessed by one-way or two-way ANOVA. When the ANOVA indicated significant differences, Fisher's *post hoc* test was used to identify differences between individual treatment groups. Student's *t* test was used when only two groups were being compared. Differences were considered significant when $p < 0.05$.

The above data show that metastin delivered directly into the lateral cerebral ventricle stimulates LH and FSH secretion. This observation supports our finding that reproduction is regulated by a permissive kisspeptin/GPR54 pathway,

Administration of metastin to monkeys

To further support our finding that the metastin receptor can regulate GnRH release, metastin was administed to rhesus monkeys. Four juvenile (16.75 to 22.75 months of age and 2.9 to 3.9 kg body weight, at the time of initiation of experiments) orchidectomized rhesus monkeys (*Macaca mulatta*) were used for the *in vivo* study. The animals were bilaterally castrated 1.25 to 2.25 months before start of the experiments. The animals were housed in individual cages and maintained under a controlled photoperiod (lights on 0700-1900) and temperature (20°C), and fed daily a high protein monkey diet at approximately 1100 h supplemented with fruit in the afternoon. Drinking water was available ad libitum. (The animals were maintained

according to the National Institutes of health Guide for Care and Use of Laboratory Animals and the protocols were approved by the Institutional Animal Care and Use Committee.)

The animals were implanted with an indwelling jugular catheter and fitted with a jacket and tether system (Suter et al., Endocrinology 139:2774-2783, 1998) for administration of an intermittent iv infusion of GnRH and for obtaining sequential blood samples. The animals were first subjected to a chronic pulsatile iv infusion of GnRH (0.3 μg every hour) for the purpose of enhancing the responsiveness of gonadotropes to GnRH stimulation to use pituitary LH secretion as a bioassay for endogenous release of GnRH (Suter et al., Endocrinology 139:2774-2783, 1998). Generally, it takes 3-4 weeks of pulsatile GnRH stimulation of the juvenile pituitary to increase plasma LH concentrations to levels approaching those observed in adults. Circulating LH levels fall abruptly to undetectable concentrations following cessation of the GnRH priming infusion. However, the response of the pituitary to GnRH is maintained for several days. In the present study, the GnRH priming infusion was stopped at least 3 days before testing the effect of kisspeptin-1, and subsequently re-initiated between the experiments to maintain pituitary responsiveness to GnRH.

To administer metastin centrally, monkeys were implanted with a 22-gauge stainless steel cannula in the lateral cerebroventricle (icv) 2-4 weeks before start of the experiments (Shahab et al., J. Neuroendorinol. 15:965-970, 2003). Orchidectomies and iv and ventricular cannulations were performed using standard aseptic surgical procedures.

Unrestrained and unsedated animals were administered vehicle (7.5% DMSO in aCSF), 30 μg metastin, and 100 μg metastin in 200 μl volume introduced slowly into the icv line via a 3-way connector above the swivel device and was immediately chased into the cerebroventricular system with 450-570 μl aCSF: a volume in slight excess of the dead space of the icv catheters (Figures 9A and 9B). Icv challenges were given separately on 2 consecutive days while the third one was given at least 10 days later. On a separate occasion, animals were administered acyline sc (60 μg/kg in morning and 120 μg/kg in late afternoon) and the next day, were challenged with icv 100 μg metastin. All icv injections were given between 0900 to 0915 h. Blood

samples were obtained at 30 min intervals for 30 min before icv injections (-30 and 0 min) and for 240 min thereafter. On some occasions, animals were also administered an icv bolus of 1229U91 an NPY receptor antagonist/agonist dissolved in aCSF (200 μg; GlaxoSmithKline, Research Triangle Park, NC) and an iv bolus of GnRH (0.3 μg) to confirm patency of the icv cannula and responsivity of gonadotropes, respectively. Additional samples were collected 30 min post 1229U91 and 15 min post GnRH.

Plasma LH concentrations were measured with a double antibody RIA system that employs recombinant cynomolgus LH (AFP342994) as standard and radioiodinated tracer, and a rabbit polyclonal antiserum to recombinant LH (AFP342994) as first antibody. The RIA reagents were provided by the National Hormone and Peptide Program. The sensitivity of the assay varied from 0.06 to 0.19 ng/ml and the intra- and inter-assay coefficients of variation were <3% and <6%, respectively.

The above experiments were carried out using the following materials and methods.

Family Kindred

A large family from Saudi Arabia with 3 first-cousin marriages came to medical attention due to infertility (Bo-Abbas et al., "Autosomal recessive idiopathic hypogonadotropic hypogonadism: genetic analysis excludes mutations in the GnRH and GnRH receptor genes," J. Clin. Endocrinol. Metab. 88(6):2730-2737, 2003). Six of the 19 total offspring from these 3 consanguineous marriages had IHH. The affected individuals (4 men, 2 women) met standard diagnostic criteria (inappropriately low gonadotropins in the presence of prepubertal levels of sex steroids, normal anterior pituitary function, and normal brain imaging), and demonstrated responsiveness to exogenous, pulsatile GnRH.

Mutation Analysis

The collection of blood samples for genetic studies was approved by the Subcommittee on Human Studies of the Massachusetts General Hospital, and all participants provided informed consent. A genome scan was performed using the ABI Prism linkage mapping primer set on 21 individuals from the Saudi family, including the 6 affected individuals (Applied Biosystems, Foster City, CA). Two-point LOD score analysis was performed using a completely penetrant autosomal recessive disease model and a gene frequency of 0.001. Linkage was demonstrated on chromosome 19p13.3 between the markers *rs7815* and *REU1905* with a maximum two point LOD score of 5.17. A shared haplotype spanning a 1.06 Mb interval was found to segregate with the disease, containing 23 known genes and an additional 49 UniGene clusters.

Mutation analysis of candidate genes, beginning with *GPR54*, was initiated using DNA extracted from whole blood. The sequence of *GPR54* cDNA (GenBank Accession number AY253981) was aligned against the published genomic sequence (Human Genome Browser; "HGB", November 2002 Assembly) to identify the genomic structure. The primers used for PCR amplification of exons were:
GPR54-e1F: 5'-GCTGGGTGAATAGAGGGC-3' (SEQ ID NO:1),
GPR54-e1R: 5'-GGAGTTTGCGACCTCTAGC-3' (SEQ ID NO:2);
GPR54-e2F: 5'-CCATCCTGCTGGTCACTCG-3' (SEQ ID NO:3);
GPR54-e2R: 5'-CACTGCGGAGCGCACTCC-3' (SEQ ID NO:4);
GPR54-e3F: 5'-GCCTGAGTGTTCGCACACG-3' (SEQ ID NO:5),
GPR54-e3R: 5'-GCGCCCATTTTCCAGATGC-3' (SEQ ID NO:6);
GPR54-e4F: 5'-GCATCTGGAAAATGGGCGC-3' (SEQ ID NO:7);
GPR54-e4R: 5'-GGAAGGCGTAGAGCAGCG-3' (SEQ ID NO:8);
GPR54-e5F: 5'-GGAGGACAGCAAGGCTGG-3' (SEQ ID NO:9); and
GPR54-e5R: 5'-AAACTGCACCGAACGTCACA-3' (SEQ ID NO:10).
Exons 1, 2, 4, and 5 were amplified using the GC-rich PCR System with its accompanying PCR program (Roche Diagnostics, Indianapolis, IN). PCR conditions for each exon were as recommended by the manufacturer with the following

modifications: exons 1, 2, and 4 for 45 sec cycles; exon 5 for 40 sec cycles. Exon 3 was amplified using standard procedures. Sequencing was performed using an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA).

Additional Populations

To determine if the observed base pair changes in *GPR54* were normal variants, a control population of 80 North American (primarily anonymous blood donors) otherwise normal volunteers was established and screened (n =160 chromosomes). 50 Middle Eastern and 50 African Americans were also screened. Additional patients with normosmic IHH (n=63) and anosmic IHH (Kallmann syndrome, n=20), diagnosed by previously published criteria (Seminara et al., "Gonadotropin-releasing hormone deficiency in the human (idiopathic hypogonadotropic hypogonadism and Kallmann's syndrome): pathophysiological and genetic considerations," Endocr. Rev. 19:521-539, 1998), were selected for further screening of *GPR54* by coding sequence analysis and RT PCR. These patients were negative for coding defects in *GNRHR* (Beranova et al., "Prevalence, phenotypic spectrum, and modes of inheritance of GnRH receptor mutations in idiopathic hypogonadotropic hypogonadism," J. Clin. Endocrinol. Metab. 86:1580-1588, 2001). Six patients with normosmic IHH who (1) had performed dose response studies to exogenous, pulsatile GnRH and (2) were negative for *GPR54* mutations by genomic screening were selected for genotype/phenotype comparisons.

Allele Specific Cloning

To demonstrate that base pair changes existed on separate alleles, the following primers were designed:

GPR54asF: 5'-CGAGGGGATGAGGCTGAGC-3' (SEQ ID NO:11); and

GPR54asR: 5'-CAAACTTCACAACGAAACTGG-3' (SEQ ID NO:12).

The PCR product was cleaned and cloned into the pCRIII-TOPO plasmid vector using the TOPO TA cloning system (Invitrogen, Carlsbad, CA). Twenty colonies were picked, grown, and the DNA from these colonies was sequenced using the same amplification primers.

RT PCR

Subjects in whom coding sequence changes were identified in GPR54 were further screened by RT PCR to rule our cryptic splice events. Total RNA from lymphoblast cell lines was extracted using the RNeasy Midi/Maxi kit (Qiagen, Valencia, CA). mRNA was reverse transcribed and overlapping sections of *GPR54* were amplified and sequenced using the following pairs of external and internal (nested) primers:

GPR54-extAF: 5'-CTCTGGACCCTGCGGACC-3' (SEQ ID NO:13);

GPR54-extAR: 5'-CAGGTGGCGCAGCATGGC-3' (SEQ ID NO:14);

GPR54-intA1F: 5'-AGCCCCTTCCTGAGTTCCA-3' (SEQ ID NO:15);

GPR54-intA1R: 5'-CGGTCAGAGTGGCACACG-3' (SEQ ID NO:16);

GPR54-intA2F: 5'-CGTGACCTTCCTCCTGTGC-3' (SEQ ID NO:17);

GPR54-intA2R: 5'-GGTGACAGGCGGTGCAGG-3' (SEQ ID NO:18);

GPR54-extBF: 5'-CGACTTCATGTGCAAGTTCG-3' (SEQ ID NO:19);

GPR54-extBR: 5'-AAACTGCACCGAACGTCACA-3' (SEQ ID NO:20);

GPR54-intB1F: 5'-GCCATGAGTGTGGACCGC-3' (SEQ ID NO:21);

GPR54-intB1R: 5'-CCAGGAACAGCTGGATGG-3' (SEQ ID NO:22);

GPR54-intB2F: 5'-CGCCTACTGCAGTGAGGC-3' (SEQ ID NO:23); and

GPR54-intB2R: 5'-CAGAAGAATAGCCGCTGTTCC-3' (SEQ ID NO:24).

All PCR reactions required the use of the GC-rich PCR System (Roche Diagnostics, Indianapolis, IN). PCR conditions were the manufacturer's standard with the following exceptions: (1) external fragments A and B required elongation of 1.5 min within each amplification cycle and (2) internal fragments A1 and B2 required 40 sec cycles.

RT primer set #1 was designed to amplify a 459 base pair segment of cDNA with a forward primer 5' to the polyA site and a reverse primer 3' to the polyA site (*incorporating* the polyA site):

GPR54-pre-polyA F: CTGTCCAAGATCAACTGTGG (SEQ ID NO:25); and

GPR54-post-polyA R: GGGATCACTTAAGGTCAGG (SEQ ID NO:26).

RT primer set #2 was designed to amplify a 174 base pair segment of cDNA with both primers located 3' to the polyA site (*downstream* from the polyA site):

GPR54-post-polyA F: GCTGACTGCAACCTCTGC (SEQ ID NO:27); and
GPR54-post-polyA R: GGGATCACTTAAGGTCAGG (SEQ ID NO:28).
PCR reactions required use of the GC-rich PCR System (Roche Diagnostics, Indianapolis, IN) with an annealing temperature of 60°C.

Generation of Mutant Constructs

The mammalian expression vector pCMVsport 6 containing WT *GPR54* (GenBank AY253982) was confirmed by direct sequencing and contained a polyA tail (Invitrogen, Carlsbad, CA). Site directed mutagenesis was performed to introduce the three mutations (L148S, R331X, and X399R) into this expression vector. In addition, a stop codon was introduced immediately after the polyA tails (constructs entitled "X399R polyA stop"). The boldface, underlined nucleotides designated in the following primers represent the mutations being incorporated:
1) GPR54-LtoS-F: 5'-CGGTGTTCCCGT**C**GCGCGCCCTGCAC-3' (SEQ ID NO:29); and GPR54-LtoS-R: 5'-GTGCAGGGCGCGC**G**ACGGGAACACCG-3' (SEQ ID NO:30);
2) GPR54-RtoX-F: 5'-CTGGGCTCGCACTTC**T**GACAGGCCTTCCGCC-3' (SEQ ID NO:31); and
GPR54-RtoX-R: 5'-GGCGGAAGGCCTGTC**A**GAAGTGCGAGCCCAG-3' (SEQ ID NO:32); and
3) GPR54-XtoR-F: 5'-GACAACGCCCCTCTC**A**GAGCGGACCCGGTGG-3' (SEQ ID NO:33); and
GPR54-XtoR-R: 5'-CCACCGGGTCCGCTC**T**GAGAGGGGCGTTGTC-3' (SEQ ID NO:34).
All mutants were created using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Proper insertion of the mutations and the complete full-length sequence of the mutant clones were confirmed by direct sequencing.

IP Assays

A natural ligand of GPR54, kisspeptin-1 (encoded by the gene *KISS1*), has recently been identified by 3 separate groups (Ohtaki et al., "Metasis suppressor gene Kiss-1 encodes peptide ligand of a G-protein-coupled receptor," Nature 411:613-617, 2001; Muir et al., "AXOR12, a novel human G protein-coupled receptor, activated by the peptide Kiss-1," J. Biol. Chem. 276:28969-28975, 2001; Kotani et al., "The metastasis suppressor gene Kiss-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54," J. Biol. Chem. 276:34631-34636, 2001) and the highest potency agonism has been demonstrated for the C-terminal decapeptide kisspeptin-1 112-121 (Muir et al., "AXOR12, a novel human G protein-coupled receptor, activated by the peptide Kiss-1," J. Biol. Chem. 276:28969-28975, 2001). This decapeptide was synthesized by the Massachusetts General Hospital Peptide Synthesis-Protein Sequencing Core Laboratory in preparation for the *in vitro* functional assays. Stimulation of GPR54 by kisspeptin-1 112-121 has been shown to increase phospatidylinositol turnover (Kotani et al., "The metastasis suppressor gene Kiss-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54," J. Biol. Chem. 276:34631-34636, 2001).

African green monkey kidney (COS-7) cells were grown to 40-80% confluence with Dulbecco's essential medium (DMEM) containing 10% fetal bovine serum/1% penicillin-streptomycin and transiently transfected with 1.5 μg/well of each *GPR54* construct or empty vector (pCMVsport6) and plated into 6-well culture plates (PolyFect Transfection Reagent; Qiagen, Valencia, CA). After a 24 h incubation at 37°C, the medium was replaced with 1 ml inositol-free DMEM for 2 h and then 1 ml of the same medium containing 2 μCi *myo*-(2-^{3}H) inositol was added (NEN Life Science Products, Boston, MA) followed by the addition of 10 mM LiCl 15 min later. After a 16 h incubation, the cells were stimulated with 10^{-7} M kisspeptin-1 112-121 for 45 min. Cells were extracted with 20 mM formic acid for 30 min X 2 and lysates were neutralized to pH 7.5. The samples were centrifuged at 14,000 x g, and the supernatants were loaded onto Ag-X8 resin anion exchange columns (Bio-Rad Laboratories, Inc., Hercules, CA). The columns were washed with 5 ml ddH_2O, 5 ml of 5 mM borax, and 60 mM sodium formate. Inositol phosphate was extracted with 3

ml of 0.9mM ammonium formate and 0.1 M formic acid. The incorporation of radioactivity in the eluates was measured in a scintillation counter, and each sample was corrected for protein content. All assay points were performed in triplicate or quadruplicate, and each experiment was repeated three times.

Peptides and reagents

Metastin (KiSS-1 (68-119)-NH2 (mouse)/metastin (1-52)) was purchased from Phoenix Pharmaceutical, Belmont, CA. Human metastin (kisspeptin-1 112-121) was synthesized at the Peptide/Protein Core Facility of the Massachusetts General Hospital Endocrine/Reproductive Endocrine Unit. For experiments involving the administration of metastin to monkeys, metastin was dissolved in monkey artificial cerebrospinal fluid (aCSF; GIBCO BRL, Life Technologies, Grand Island, NY) containing 15% DMSO (Sigma Chemical Co., St. Louis, MO). The working metastin solution contained 7.5% DMSO. GnRH (GMP-26 code no. 230-110-40) and acyline were synthesized at the Salk institute (Contract N01-HD-0-2906) and Bioqual (Rockville, MD), respectively and made available by the Contraception and Reproductive Health Branch of the Center for Population Research at NICHD. GnRH was dissolved in saline and acyline was dissolved in 5% aqueous mannitol.

Quantitative RT PCR

Quantitative RT PCR was performed on RNA isolated from immortalized patient lymphoblasts using the Taqman One-Step RT PCR Master Mix (Applied Biosystems, Foster City, CA). Different primers and probes capable of amplifying the R331X and X399R alleles selectively were designed and synthesized. Samples were run in quadruplicate in a minimum of two independent experiments. The β actin gene was used as an endogenous control to standardize for expression levels.

Genotype-Phenotype Correlations

The patient carrying mutations R331X and X399R was admitted to the General Clinical Research Center (GCRC) of the Massachusetts General Hospital (MGH). Frequent blood sampling was performed every 10 min for 12 h for

measurement of LH and FSH. The patient was then placed on exogenous, pulsatile GnRH administered subcutaneously at 2 hr intervals and his dose titrated as an outpatient until his pituitary-gonadal axis had normalized. After 11 months of pulsatile GnRH therapy, the patient was re-admitted to the GCRC for a dose response study, in which 4 doses of GnRH spanning 1.5 log orders were administered IV (7.5-250 ng/kg/bolus). The GnRH doses were administered in randomized order to assure that priming was not determining the resultant gonadotrope responses. Frequent LH sampling occurred after each dose. Pulsatile hormone secretion was assessed using the modified version of the Santen and Bardin method (Filicori et al., J. Clin. Endocrinol. Metab. 62:1136-1144, 1986; and Santen and Bardin, J. Clin. Invest. 52:2617-2628, 1973). These clinical studies were approved by the Subcommittee on Human Studies of MGH and the patient provided written informed consent.

Clinical Assays

Serum LH and FSH concentrations were determined by immunoassays calibrated against the Second International Reference Preparation of human Menopausal Gonadotropin; WHO 71/223 (Crowley et al., "The biologic activity of a potent analogue of gonadotropin-releasing hormone in normal and hypogonadotropic men," N. Engl. J. Med. 302:1052-1057, 1980; Filicori et al., "Neuroendocrine regulation of the corpus luteum in the human. Evidence for pulsatile progesterone secretion," J. Clin. Invest. 73:1638-1647, 1984; Welt et al., "Inhibin A and inhibin B response to gonadotropin withdrawal depends on stage of follicle development," J. Clin. Endocrinol. Metab. 84:2163-2169, 1999), with a minimal detectable dose (MDD) of 0.8 IU (hMG, WHO 71/223)/L. Inter- and intra-assay coefficients of variation were less than 10%.

Determination of the distribution of *KiSS-1* mRNA in the hypothalamus of the mouse

Tissue Preparation

Mice were anesthetized with isoflurane and then killed by decapitation. Brains were removed and frozen on dry ice. Sections in the coronal plane (20 μm) were cut on a cryostat, thaw-mounted onto SuperFrost Plus slides (VWR Scientific, West Chester, PA), and stored at -80°C. Sections were collected from the diagonal band of Broca to the mammillary bodies.

Cloning of partial cDNA for mouse KiSS-1

Total RNA was extracted from mouse brain using an RNAqueous Kit (Ambion, Inc., Austin, TX). RNA was reverse transcribed into cDNA with a RetroScript kit (Ambion) primed with oligodeoxythymidine (dT) for subsequent PCR. Primers were designed based on the published sequence of the *KiSS-1* mouse gene (Genbank accession number AF472576) with forward primers corresponding to bases 76 - 93 and reverse primers corresponding to bases 466-486. Primers were custom synthesized (QIAGEN, Valencia, CA). PCR reactions contained the following in a volume of 20 μl: 2 μl of reverse transcriptase reaction product; 0.2 μM of each primer; 12.5 μl RediTaq polymerase (Sigma-Aldrich, Natick, MA); and 8.5 μl of water. Reactions were performed in a PTC-100 thermal cycler (MJ Research, Inc., Watertown, MA) using the following protocol: cDNA was denatured for 2 minutes at 94°C, then 35 cycles were carried out at 94°C for 1 min, 55°C for 1.5 min, and 72°C for 2 min, with a final 5 min extention at 72°C. After electrophoresis on a 2% agarose (wt/vol) gel, a single DNA fragment was obtained of approximately the expected size (411 bp) and gel purified with a QiaQuick gel extraction kit (QIAGEN). The PCR product was confirmed to be the mouse KiSS-1 probe by sequencing and was cloned into the pAMP1 plasmid (Gibco/Invitrogen, Carlsbad, CA).

In situ hybridization

Antisense and sense mouse KiSS-1 probes were transcribed from linearized pAMP1 plasmid containing the mouse KiSS-1 insert with T7 and SP6 Polymerase Plus (Ambion), respectively. Radiolabeled probes were synthesized *in vitro* by

inclusion of the following ingredients in a volume of 20 µl: 250 µCi 33P-UTP (Perkin Elmer Life Sciences, Boston, MA); 1 µg linearized DNA; 0.5 mM each ATP, CTP, GTP; 40 U polymerase. Residual DNA was digested with 4 U DNase (Ambion) and the DNase reaction was terminated by addition of 2 µl of 0.5 M EDTA (pH = 8.0). The riboprobes were separated from unincorporated nucleotides with NucAway Spin Columns (Ambion).

Slides with mouse hypothalamic sections from three adult male C57BL/6 were processed before hybridization as previously reported (Cunningham et al., Endocrinology 143: 755-763, 2002). Radiolabeled antisense and sense KiSS-1 riboprobes were denatured, dissolved in hybridization solution at a concentration of 0.1 pmol/ml along with tRNA (1.9mg/ml) and applied to slides. Two negative controls were used to demonstrate specificity of the KiSS-1 riboprobe: slides were incubated with radiolabeled antisense probe in the presence of excess (500 X) unlabeled antisense probe, or with an equivalent concentration of radiolabeled sense KiSS-1 probe. Slides were covered with glass coverslips, placed in a humid chamber, and incubated overnight at 55°C. The following day, slides were washed as previously reported (Cunningham et al., Endocrinology 143: 755-763, 2002). Slides were then dipped in NTB-3 liquid emulsion (Eastman Kodak Co., Rochester, NY). Slides were developed 3 days later, and coverslips were applied.

Animals

The transgenic mice (*Gpr54* ^{tm1PTL}) were maintained as an inbred stock on a 129S6/SvEv genetic background. The targeting strategy engineered a germline deletion of transmembrane loops 1 and 2 and encompassing domains (Figure 6A). Correct targeting was verified for 3' and 5' arms by Southern blot and PCR. All experiments were performed under authority of a UK Home Office Project License and approved by a local ethical panel.

For experiments to determine the role of kisspeptins in the regulation of gonadotrophin secretion in the mouse, adult male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were individually housed and were maintained on a 12 h light, 12 h dark cycle (lights on at 0600 h). All animals had access to standard rodent

chow and water *ad libitum*. All procedures were approved by the Animal Care Committee of the School of Medicine of the University of Washington in accordance with the NIH Guide for Care and Use of Laboratory Animals.

Gonadotropin Releasing Hormone Injection

Wild-type female animals were staged by vaginal smearing. Wild-type females at diestrous, and GPR54 -/- females received 4 intraperitoneal injections of 25 ng gonadotropin releasing hormone at 30 min intervals (Collins et al., Neurobiology of Aging 2:125-131, 1981). Animals were killed 30 min after the last injection. Blood and pituitary samples were treated as described in Collins et al. (Neurobiology of Aging 2:125-131, 1981) except the pituitary was homogenized in 0.3 ml phosphate buffered saline.

Mouse Hormone Assays

The luteinizing hormone (LH) IRMA and follicle stimulating hormone (FSH) RIA sensitivities were 0.07 and 2 ng/ml respectively (intra-assay variation 6.0 percent and 10 percent, inter-assay variation 12.5 percent and 18 percent). Gonadotropin releasing hormone was measured by RIA with a detection limit of 0.2 pg/tube (0.83 pg/ml) and an intra-assay variation of 13 percent (see, e.g., Caraty et al., Endocrinology 136:3452-3460, 1995). Testosterone was measured by RIA with a sensitivity of 0.2 nmol/L (intra-assay variation 6.0 percent, inter-assay variation 18 percent). 17β estradiol was measured by ELISA with a sensitivity of 10 pg/ml (intra-assay variation 3.9 percent, inter-assay variation 10 percent).

Histology

Tissues were dissected and fixed for 4 h in 4 percent formaldehyde followed by 3 washes in 0.01 phosphate buffered saline. Ovaries, testes, and adrenal glands were wax embedded and sectioned a 3-4 μm. Tissue sections were stained by haemotoxylin and eosin. Mammary glands were dissected and fixed for 2-4 h at 23°C in fixative (absolute ethanol:chloroform:glacial acetic acid=6:3:1), washed in 70 percent ethanol for 15 min, re-hydrated, and stained overnight in Carmine Alum Stain

in 500 ml distilled water and boiled for 20 min. Slides were washed in increasing concentrations of ethanol (70 percent, 95 percent, and 100 percent) for 15 min each, cleared in xylene for 30 min, and mounted.

Example 2

Screens for Compounds that Alter GPR54 Biological Activity

GPR54 nucleic acid and amino acid sequences may be used to identify compounds that alter a biological activity of GPR54. Such compounds may be used in the treatment or diagnosis of a GPR54 related disorder, e.g., idiopathic hypogonadotropic hypogonadism, amenorrhea, delayed puberty, microphallus, microspadius, central precocious puberty, polycystic ovarian disease, delayed puberty, prostate cancer, preparation for *in vitro* fertilization, endometriosis, uterine fibroids, and infertility. In addition, compounds identified using the methods described herein may be used as contraceptives.

Assays to be used for identifying and/or characterizing compounds that alter a GPR54 biological activity may include measuring intracellular calcium release, phosphorylation of a polypeptide in a signaling pathway regulated by GPR54, arachidonic acid release, and phosphatidylinositol turnover. Further, the exemplary competitive binding assays described below may be used to identify a candidate compound that can be used in the claimed methods.

Test extracts and compounds

In general, compounds that alter a GPR54 biological activity are identified from large libraries of both natural products, synthetic (or semi-synthetic) extracts or chemical libraries, according to methods known in the art.

Those skilled in the art will understand that the precise source of test extracts or compounds is not critical to the screening procedure(s) of the invention. Accordingly, virtually any number of chemical extracts or compounds can be screened using the exemplary methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as

modifications of existing compounds. Numerous methods are also available for generating random or directed synthesis (e.g., semi-synthesis or total synthesis) of any number of chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and nucleic acid-based compounds. Synthetic compound libraries are commercially available from, for example, Brandon Associates (Merrimack, NH) and Aldrich Chemical (Milwaukee, WI).

Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, including, but not limited to, Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceangraphics Institute (Ft. Pierce, FL), and PharmaMar, U.S.A. (Cambridge, MA). In addition, natural and synthetically produced libraries are produced, if desired, according to methods known in the art (e.g., by combinatorial chemistry methods or standard extraction and fractionation methods). Furthermore, if desired, any library or compound may be readily modified using standard chemical, physical, or biochemical methods.

In addition, those skilled in the art readily understand that methods for dereplication (e.g., taxonomic dereplication, biological dereplication, and chemical dereplication, or any combination thereof) or the elimination of replicates or repeats of materials already known for their effects on compounds associated with estrogen regulation should be employed whenever possible.

When a crude extract is found to alter a GPR54 biological activity, further fractionation of the positive lead extract is necessary to isolate chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and identification of a chemical entity within the crude extract having activities that alter a GPR54 biological activity. The same *in vivo* and *in vitro* assays described herein for the detection of activities in mixtures of compounds can be used to purify the active component and to test derivatives thereof. Methods of fractionation and purification of such heterogenous extracts are known in the art.

GPR54 Binding Assays

A compound that binds GPR54 and/or competes with a known ligand for GPR54, for example a peptide derived from Kisspeptin (e.g., kisspeptin-54, kisspeptin-14, kisspeptin-13, kisspeptin-10, or metastin) is a candidate compound that may be used to treat or diagnose a GPR54 related disorder. Such a compound may be identified by contacting a cell with a test compound in the presence of a kisspeptin polypeptide and assaying for a decrease of GnRH secretion or action, relative to a control cell in the presence of said kisspeptin polypeptide and not contacted with said test compound. A saturation-binding assay to determine the K_d value of the known ligand first may be performed. In these assays, the known ligand may be, for example, ^{125}I-kisspeptin-10. The known ligand can be used as a competitor for an unlabeled candidate compound. For instance, a reduction in ^{125}I-kisspeptin-10 binding in the presence of the candidate compound, when compared to the K_d value determined in a saturation binding experiment, is indicative that the candidate compound competitively binds GPR54. A compound that binds competitively to GPR54 may have a K_i of about, e.g., 1.0-5.0. Desirably, the K_i of a known ligand may be approximately 1.4, 1.6, 2.3, or 4.2.

Similarly, a compound that binds GPR54 may be a candidate compound that can be used to treat or diagnose a GPR54 related disorder. *In vitro* protein binding assays are standard in the art and may include, e.g., the use of an epitope-tagged GPR54 polypeptide. Examples of commercially available epitope tags include, His-tags, HA-tags, FLAG®-tags, and c-Myc-tags. However, any epitope that is recognized by a polypeptide, such as an antibody, also may be used as an epitope tag. See, for example, Ausubel et al., *Current Protocols in Molecular Biology*, Wiley Interscience, New York, 2001; and Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, N.Y., (1989). For instance, an epitope-tagged GPR54 protein used in *in vitro* binding experiments may be expressed in CHO cells. Polypeptides that bind GPR54 may be purified on protein gels and, further, may be identified by end-sequencing.

Furthermore, candidate compounds may be identified at the level of GPR54 polypeptide production using standard immunological techniques, such as Western blotting or immunoprecipitation with an antibody specific for a GPR54 polypeptide. For example, immunoassays may be used to detect or monitor the expression of a GPR54 polypeptide in an organism, tissue, or a cell line. Exemplary cell lines that may be used in these assays include GT1-7 (Mellon et al., Neuron 5:1-10, 1990); GnV-3 (Castillo et al., The Endocrine Society's 84th Annual Meeting, San Francisco, CA, Abstract OR45-2, 2002); and GN-11 (Zakaria et al., Mol. Endocrinol. 10:1282-1291, 1996). Polyclonal or monoclonal antibodies that are capable of binding to GPR54 may be used in any standard immunoassay format (e.g., ELISA, Western blot, or RIA assay) to measure the level of the polypeptide. In some embodiments, a compound that promotes an increase or decrease in the expression of a GPR54 polypeptide, or of a component of a GPR54 signaling pathway, is a candidate or test compound that may be used in the treatment or diagnosis of the reproductive disorders described herein, as well as in contraception and *in vitro* fertilization.

Alternatively, the expression of a GPR54 nucleic acid molecule may be measured, for example, by microarray analysis, Northern blot analysis (Ausubel et al., *Current Protocols in Molecular Biology*, Wiley Interscience, New York, 2001), or RT-PCR, using any appropriate fragment prepared from the nucleic acid molecule as a hybridization probe. The level of GPR54 nucleic acid molecule expression in the presence of the candidate compound may be compared to the level measured in a control culture medium lacking the candidate molecule. A compound which promotes an increase or a decrease in the expression of a GPR54 nucleic acid molecule or of a component of a GPR54 signaling pathway, is a candidate or test compound that may be used in the treatment or diagnosis of the reproductive disorders described herein, as well as in contraception and *in vitro* fertilization.

Moreover, a GPR54 nucleic acid molecule that includes a regulatory sequence may be expressed as a transcriptional or translational fusion with a detectable reporter, and expressed in a cell line. The cell expressing the fusion protein is then contacted with a candidate compound, and the expression of the detectable reporter in that cell is compared to the expression of the detectable reporter in an untreated control cell. A

candidate compound that alters (e.g., increases or decreases) the expression of the detectable reporter, e.g., luciferase, is a compound that may be useful for the diagnosis or treatment of a reproductive order described herein, as well as in contraception and *in vitro* fertilization.

Example 3

Biological Activity Assays

A compound that binds GPR54, or that alters the expression level of a GPR54 polypeptide or nucleic acid molecule, may be further screened to determine whether the compound alters a biological activity of GPR54, e.g., affects GnRH secretion. Such a compound may either decrease secretion of GnRH and cause hypogonadotropic hypogonadism as a therapy for various medical conditions such as prostate cancer, endometriosis, central precocious puberty, IVF, uterine fibroids, polycystic ovarian disease, and for contraception or restore normal pulsatile GnRH secretion in disorders in which it is abnormal. Since GPR54's normal function is to modulate and support GnRH secretion and mutations in this receptor cause a form of hypogonadotropic hypogonadism, antagonists to GPR54 will result in a similar hypogonadotropic state, and, thus, may be used to treat central precocious puberty, prostate cancer, endometriosis, uterine fibroids, contraception, and as a preparation for in vitro fertilization. Moreover, agonist compounds identified using the methods of the invention, i.e., screening for agonist activity in a GPR 54-based methodology may be used in treatments for delayed puberty, infertility, such as for amenorrhea, and for identification of novel contraceptives. Such compounds may be identified using a number of standard methods in the art, including the following exemplary assays.

Intracellular Calcium Release Assay

An exemplary method to determine whether a compound alters a biological activity of a GPR54 polypeptide is an intracellular calcium release assay. In such assays mammalian cells, e.g., Chinese Hamster Ovary (CHO) cells, can be pretreated with 100 ng/ml pertussis toxin for 15 hours and incubated in Hanks' balanced salt medium containing 0.1% bovine serum albumin and 2.8 μg/ml Fura-2, a UV light-

excitable, ratiometric Ca^{2+} indicator (Molecular Probes), at 37°C for 45 min in the presence or absence of a candidate compound. Upon binding Ca^{2+}, Fura-2 exhibits an absorption shift that can be observed by scanning the excitation spectrum between 300 and 400 nm, while monitoring the emission at ~510 nm. Fura-2 exhibits K_d values that are close to typical basal Ca^{2+} levels in mammalian cells (~100 nM), and displays high selectivity for Ca^{2+} binding relative to Mg^{2+} (see also, Kotani et al. (Br. J. Pharmacol. 133:138-144, 2001)). For this assay, the cells may be at a density of 10^5/ml and intracellular Ca^{2+} concentration can be measured using a luminescence spectrophotometer, e.g., a LS50B spectrophotometer (PerkinElmer Life Sciences).

Phosphorylation Assays

Further, whether a compound alters a biological activity of a GPR54 polypeptide may be determined using assays that determine the phosphorylation state of proteins in a GPR54-regulated signaling pathway. For example, an alteration in the phosphorylation state of a downstream component of a GPR54-regulated signaling pathway is indicative of an alteration in the biological activity of a GPR54 polypeptide. Proteins that may be phosphorylated in a GPR54-regulated signaling pathway include focal adhesion kinase (FAK), paxillin, MAP kinases ERK1 and ERK2, PKA, PKC, and p38 MAP kinase (Kotani et al., Br. J. Pharmacol. 133:138-144, 2001; Kotani et al. J. Biol. Chem. 276:34631-34636, 2001). Phosphorylation assays may be carried out by plating a cell line (e.g., 4 x 10^6 CHO cells in Dulbecco's modified Eagle medium (DMEM) tissue culture medium) onto collagen-IV-coated 6 cm dishes and incubating these dishes for 2 h at 37°C before contacting the cells with a compound that binds GPR54. The cells can then be lysed with 1 ml of lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EGTA, 2 mM Na_3VO_4, 50 mM NaF, 1% NP-40, 4 mM $Na_4P_2O_7$ and protease inhibitors at pH 7.4). Each 0.45 ml of lysate can then be precipitated with an antibody against the potentially phosphorylated protein (e.g., FAK, paxillin, ERK1, ERK2, or p38 MAP kinase) using G-sepharose. One half of the precipitate may be Western blotted using ECL phosphorylation detection (Amersham Pharmacia), and the other half may be Western blotted with an antibody against the potentially phosphorylated protein.

Phosphatidylinositol Turnover

Monitoring phosphatidylinositol turnover may also be used to determine whether a compound alters a biological activity of a GPR54 polypeptide. For example, a mammalian cell line (e.g. CHO cells) may be cultured overnight in DMEM containing 5% fetal calf serum (FCS) and 1 μCi/ml *myo*-[^{3}H]inositol (Amersham Pharmacia Biotech). The cells can then be detached from the tissue culture plate with phosphate-buffered saline-EDTA, washed, and incubated for 20 min. at 37°C with a candidate compound in 500 μl of 25 mM Tris-HCl, pH 7.4, 121.5 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 15 mM glucose, and 10 mM LiCl. The incubation can be terminated with 500 μl of buffer containing 88% methanol and 0.12 M HCl. The cells may then be filtered through Whatman GF/B filters and washed with ice-cold buffer. Bound radioactivity can be measured in a TRI-CARB 2100TR counter (Packard Instrument Co.).

Arachidonic Acid Release Assay

One skilled in the art may also use an arachidonic release assay to determine whether a compound alters a biological activity of a GPR54 polypeptide. For instance, a mammalian cell line, e.g., CHO cells, may be cultured at a density of 2.5 x 10^6 cells/ml in 6-well plates overnight in 0.1 μCi/ml [^{3}H] arachidonic acid (Amersham Pharmacia Biotech), washed, and incubated for 60 min. at 37°C with a candidate compound in DMEM containing 0.1% bovine serum albumin (BSA). The bound radioactivity can then be compared to a control which was incubated under the same conditions, except in the absence of the candidate compound. Bound radioactivity may be measured, for example, using a TRI-CARB 2100TR counter (Packard Instrument Co.).

Example 4

Administration of Compounds that Alter GPR54 Biological Activity

The compounds identified using the claimed methods may be administered by any suitable means that result in a concentration that alters the biological activity of a GPR54 polypeptide upon reaching the target region. The following methods of

administration are suitable for treating reproductive disorders, such as those described herein, as well as for contraception. Suitable compounds include antagonists which decrease the biological activity of a GPR54 polypeptide and agonists which can be used to desensitize GPR54 and, thereby, reduce its biological activity.

The therapeutic compound may be contained in any appropriate amount in any suitable carrier substance, and is generally present in an amount of 1-95% by weight of the total weight of the composition. The composition may be provided in a dosage form that is suitable for an oral or parenteral (e.g., subcutaneous, intravenous, intramuscular, or intraperitoneal), buccal, transdermal, or transvaginal administration route. The pharmaceutical compositions may be formulated according to conventional pharmaceutical practice (see, e.g., Remington: The Science and Practice of Pharmacy (20th ed.), ed. A.R. Gennaro, Lippincott Williams & Wilkins, 2000 and Encyclopedia of Pharmaceutical Technology, eds. J. Swarbrick and J. C. Boylan, 1988-1999, Marcel Dekker, New York). The pharmaceutical composition may be administered orally or parenterally by injection, infusion or implantation (subcutaneous, intravenous, intramuscular, intraperitoneal, or the like) in dosage forms, formulations, or via suitable delivery devices or implants containing conventional, non-toxic pharmaceutically acceptable carriers, e.g., ethyl vinyl acetate, and adjuvants. The formulation and preparation of such compositions are well known to those skilled in the art of pharmaceutical formulation. Formulations can be found in Remington: The Science and Practice of Pharmacy, *supra*.

Formulations for oral use include tablets containing the active ingredient(s) in a mixture with non-toxic pharmaceutically acceptable excipients, and such formulations are known to the skilled artisan (e.g., U.S. Patent Serial Nos.: 5,817,307, 5,824,300, 5,830,456, 5,846,526, 5,882,640, 5,910,304, 6,036,949, 6,036,949, 6,372,218, hereby incorporated by reference). These excipients may be, for example, inert diluents or fillers (e.g., sucrose, sorbitol, sugar, mannitol, microcrystalline cellulose, starches including potato starch, calcium carbonate, sodium chloride, lactose, calcium phosphate, calcium sulfate, or sodium phosphate); granulating and disintegrating agents (e.g., cellulose derivatives including microcrystalline cellulose, starches including potato starch, croscarmellose sodium, alginates, or alginic acid);

binding agents (e.g., sucrose, glucose, sorbitol, acacia, alginic acid, sodium alginate, gelatin, starch, pregelatinized starch, microcrystalline cellulose, magnesium aluminum silicate, carboxymethylcellulose sodium, methylcellulose, hydroxypropyl methylcellulose, ethylcellulose, polyvinylpyrrolidone, or polyethylene glycol); and lubricating agents, glidants, and anti-adhesives (e.g., magnesium stearate, zinc stearate, stearic acid, silicas, hydrogenated vegetable oils, or talc). Other pharmaceutically acceptable excipients can be, colorants, flavoring agents, plasticizers, humectants, buffering agents, and the like.

The tablets may be uncoated or they may be coated by known techniques, optionally to delay disintegration and absorption in the gastrointestinal tract and thereby providing a sustained action over a longer period. The coating may be adapted to release the active compound in a predetermined pattern (e.g., in order to achieve a controlled release formulation) or it may be adapted not to release the active compound until after passage of the stomach (enteric coating). The coating may be a sugar coating, a film coating (e.g., based on hydroxypropyl methylcellulose, methylcellulose, methyl hydroxyethylcellulose, hydroxypropylcellulose, carboxymethylcellulose, acrylate copolymers, polyethylene glycols and/or polyvinylpyrrolidone), or an enteric coating (e.g., based on methacrylic acid copolymer, cellulose acetate phthalate, hydroxypropyl methylcellulose phthalate, hydroxypropyl methylcellulose acetate succinate, polyvinyl acetate phthalate, shellac, and/or ethylcellulose). Furthermore, a time delay material such as, e.g., glyceryl monostearate or glyceryl distearate may be employed.

The solid tablet compositions may include a coating adapted to protect the composition from unwanted chemical changes, (e.g., chemical degradation prior to the release of the active compound). The coating may be applied on the solid dosage form in a similar manner as that described in Encyclopedia of Pharmaceutical Technology, supra.

To active compounds may be mixed together in the tablet, or may be partitioned. In one example, the first active compound is contained on the inside of

the tablet, and the second active compound is on the outside, such that a substantial portion of the second active compound is released prior to the release of the first active compound.

Formulations for oral use may also be presented as chewable tablets, or as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent (e.g., potato starch, lactose, microcrystalline cellulose, calcium carbonate, calcium phosphate or kaolin), or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example, peanut oil, liquid paraffin, or olive oil. Powders and granulates may be prepared using the ingredients mentioned above under tablets and capsules in a conventional manner using, e.g., a mixer, a fluid bed apparatus, or spray drying equipment.

Controlled release compositions for oral use may, e.g., be constructed to release the active compound by controlling the dissolution and/or the diffusion of the active compound.

Dissolution or diffusion controlled release can be achieved by appropriate coating of a tablet, capsule, pellet, or granulate formulation of compounds, or by incorporating the compound into an appropriate matrix. A controlled release coating may include one or more of the coating substances mentioned above and/or, e.g., shellac, beeswax, glycowax, castor wax, carnauba wax, stearyl alcohol, glyceryl monostearate, glyceryl distearate, glycerol palmitostearate, ethylcellulose, acrylic resins, dl-polylactic acid, cellulose acetate butyrate, polyvinyl chloride, polyvinyl acetate, vinyl pyrrolidone, polyethylene, polymethacrylate, methylmethacrylate, 2-hydroxymethacrylate, methacrylate hydrogels, 1,3 butylene glycol, ethylene glycol methacrylate, and/or polyethylene glycols. In a controlled release matrix formulation, the matrix material may also include, e.g., hydrated metylcellulose, carnauba wax and stearyl alcohol, carbopol 934, silicone, glyceryl tristearate, methyl acrylate-methyl methacrylate, polyvinyl chloride, polyethylene, and/or halogenated fluorocarbon.

A controlled release composition containing one or more of the compounds identified using the claimed methods may also be in the form of a buoyant tablet or capsule (i.e., a tablet or capsule that, upon oral administration, floats on top of the gastric content for a certain period of time). A buoyant tablet formulation of the

compound(s) can be prepared by granulating a mixture of the active compound with excipients and 20-75% w/w of hydrocolloids, such as hydroxyethylcellulose, hydroxypropylcellulose, or hydroxypropylmethylcellulose. The obtained granules can then be compressed into tablets. On contact with the gastric juice, the tablet forms a substantially water-impermeable gel barrier around its surface. This gel barrier takes part in maintaining a density of less than one, thereby allowing the tablet to remain buoyant in the gastric juice.

With respect to the therapeutically active compounds identified using the methods of the invention, it is not intended that the administration of the claimed compound to a patient be limited to a particular mode of administration, dosage, or frequency of dosing; the present invention contemplates all modes of administration, including oral, intramuscular, intravenous, intraperitoneal, intravesicular, intraarticular, intralesional, subcutaneous, transvaginal, or buccal or any other route sufficient to provide a dose adequate to alter a biological activity of a GPR54 polypeptide. The compound(s) may be administered to the patient in a single dose or in multiple doses. When multiple doses are administered, the doses may be separated from one another by, for example, one day, two days, one week, two weeks, or one month. For example, a therapeutic compound may be administered once a week for, e.g., 2, 3, 4, 5, 6, 7, 8, 10, 15, 20, or more weeks. It is to be understood that, for any particular subject, specific dosage regimes should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions. The precise dose will vary dependent on the compound used and the rate of clearance of the polypeptide.

While the attending physician ultimately will decide the appropriate amount and dosage regimen, a therapeutically effective amount of a compound identified using the claimed method may be, for example, in the range of about 0.1 mg to 50 mg/kg body weight/day or 0.70 mg to 350 mg/kg body weight/week. Desirably a therapeutically effective amount is in the range of about 0.10 mg to 20.0 mg/kg, and more desirably in the range of about 0.20 mg to 15.0 mg/kg for example, about 0.2,

0.3, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 7.0, 8.0, 8.5, 9.0, 10.0, 11.0, 12.0, 13.0, 14.0, or 15.0 mg/kg body weight administered daily, every other day, or twice a week.

For example, a suitable dose is an amount of the therapeutic compound that, when administered as described above, is capable of increasing or decreasing the biological activity of a GPR54 polypeptide by at least 20% above or below the basal (i.e., untreated) level. In general, an appropriate dosage and treatment regimen provides the active compound(s) in an amount sufficient to provide therapeutic and/or prophylactic benefit. Such a response can be monitored by establishing an improved clinical outcome (e.g., more frequent remissions, complete or partial, or longer disease-free survival) in treated patients as compared to non-treated patients. According to this invention, the administration of the a compound identified using the claimed methods can increase a biological activity of a GPR54 polypeptide by at least 20%, 40%, 50%, or 75% above that of an untreated control. Such responses can be monitored by any standard technique known in the art including those described herein, such as calcium release assays, determining the phosphorylation state of a polypeptide in a signaling pathway regulated by GPR54, arachidonic acid release assays, and phosphatidylinositol turnover assays. More preferably, the biological activity of a GPR54 polypeptide is increased by 80%, 90%, 95%, or even 100% above that of an untreated control. Alternatively, the administration of a compound identified using the claimed methods can decrease the biological activity of a GPR54 polypeptide by at least 20%, 40%, 50%, or 75% below that of an untreated control as measured by any standard assay known in the art. More desirably, the biological activity of a GPR54 polypeptide is decreased by 80%, 90%, 95%, or even 100% below that of an untreated control. Such responses can be monitored by any standard technique known in the art including those described herein, such as calcium release assays, determining the phosphorylation state of a polypeptide in a signaling pathway regulated by GPR54, arachidonic acid release assays, and phosphatidylinositol turnover assays.

Example 5

Diagnosis of Reproductive Disorders

An alteration in a GPR54 nucleic acid or amino acid sequence may be used to diagnose a patient as having or as being at risk of acquiring a reproductive disorder, for example, IHH, amenorrhea, microphallus, hypospadius, polycystic ovarian disease, prostate cancer, endometriosis, or uterine fibroids. Exemplary alterations include a 443T>C alteration in the human GPR54 nucleic acid sequence (e.g., GenBank Accession No. AY029541, AF343725, NM_032551, or AY253981) that results in a serine for leucine substitution at position 148 of corresponding amino acid sequence, a 991C>T alteration in the human GPR54 nucleic acid sequence that results in the replacement of arginine at position 331 of the corresponding amino acid sequence with a premature stop codon, and a 1195T>A alteration in the human GPR54 nucleic acid sequence that results in the replacement of the stop codon at residue 399 of the corresponding amino acid sequence with an arginine.

For instance, probes and primers for a human GPR54 sequence may be used as markers to detect a particular alteration in a sample from a patient. An alteration in a human GPR54 gene may be identified in a biological sample obtained from a patient using a variety of methods available to those skilled in the art. Generally, these techniques involve PCR amplification of nucleic acid from the patient sample, followed by identification of the genetic lesion by either altered hybridization, aberrant electrophoretic gel migration, restriction fragment length polymorphism (RFLP) analysis, binding or cleavage mediated by mismatch binding proteins, or direct nucleic acid sequencing. Any of these techniques may be used to facilitate detection of a genetic lesion in a candidate gene, and each is well known in the art; examples of particular techniques are described, without limitation, in Orita et al. (*Proc. Natl. Acad. Sci. USA* 86:2766-2770, 1989) and Sheffield et al. (*Proc. Natl. Acad. Sci. USA* 86:232-236 (1989)). Furthermore, expression level of the candidate gene in a biological sample (e.g., a biopsy) may be monitored by standard Northern blot analysis or may be aided by PCR, including RT PCR (see, e.g., Ausubel et al., *Current Protocols in Molecular Biology*, Wiley Interscience, New York, 2001; Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 3rd edition, Cold Spring

Harbor Laboratory Press, N.Y., 2001; *PCR Technology: Principles and Applications for DNA Amplification*, H.A. Ehrlich, Ed., Stockton Press, NY; Yap et al., *Nucl. Acids. Res.* 19:4294 (1991)).

Alternatively, a GPR54 biological activity may be used to diagnose a mammal as having or being at risk of developing a reproductive disorder. For example an alteration in the biological activity of a GPR54 amino acid molecule, or a fragment thereof, relative to a control amino acid sequence is indicative that a mammal has or has a propensity for developing a reproductive disorder. Desirably, the biological activity is an alteration in the level of inositol phosphate production.

Furthermore, antibodies against a GPR54 polypeptide may be used to detect altered expression levels of a human GPR54 protein, including a lack of expression, or a change in its mobility on a gel, indicating a change in structure or size. In addition, antibodies may be used for detecting an alteration in the expression pattern or the sub-cellular localization of the protein. Such antibodies include ones that recognize both the wild-type and mutant protein, as well as ones that are specific for either the wild-type or an altered form of the protein.

Antibodies used in the methods of the invention may be produced using amino acid sequences that do not reside within highly conserved regions, and that appear likely to be antigenic, as analyzed by criteria such as those provided by the Peptide Structure Program (Genetics Computer Group Sequence Analysis Package, Program Manual for the GCG Package, Version 7, 1991) using the algorithm of Jameson and Wolf (*CABIOS* 4:181 (1988)) (See, e.g., Harlow and Lane, Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, N.Y., 1999). These fragments can be generated by standard techniques, e.g., by the PCR, and cloned into the pGEX expression vector (Ausubel et al., *Current Protocols in Molecular Biology*, Wiley Interscience, New York, 2001). GST fusion proteins are expressed in *E. coli* and purified using a glutathione agarose affinity matrix as described in Ausubel et al. (*Current Protocols in Molecular Biology*, Wiley Interscience, New York, 2001).

Other Embodiments

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure come within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth.

Seminara et al. (N. Engl. J. Med. 349:1614-1627, 2003) and all other references cited herein are hereby incorporated by reference.

What is claimed is:

CLAIMS

1. A method of identifying a candidate compound for suppressing endogenous GnRH secretion or action, said method comprising contacting a GPR54 polypeptide with a test compound and assaying a biological activity of said GPR54 polypeptide, wherein a decrease of said biological activity, relative to a control not contacted with said test compound, identifies said test compound as a candidate compound for suppressing endogenous GnRH secretion or action.

2. The method of claim 1, wherein said GPR54 polypeptide is contacted with said test compound in the presence of a kisspeptin polypeptide and wherein said control is in the presence of said kisspeptin polypeptide.

3. A method of identifying a candidate compound for suppressing endogenous GnRH secretion or action, said method comprising contacting a cell with a test compound in the presence of a kisspeptin polypeptide and assaying for a decrease of GnRH secretion or action, relative to a control cell in the presence of said kisspeptin polypeptide and not contacted with said test compound, wherein a decrease in GnRH secretion or action identifies said test compound as a candidate compound for suppressing endogenous GnRH secretion or action.

4. A method of identifying a candidate compound for treating a disorder selected from the group consisting of idiopathic hypogonadotropic hypogonadism, delayed puberty, amenorrhea, and polycystic ovarian disease, said method comprising contacting a GPR54 polypeptide with a test compound and assaying a biological activity of said GPR54 polypeptide, wherein an increase of said biological activity, relative to a control not contacted with said test compound, identifies said test compound as a candidate compound for treating idiopathic hypogonadotropic hypogonadism, delayed puberty, amenorrhea, and polycystic ovarian disease.

5. A method of identifying a candidate compound for use in treating infertility, in *in vitro* fertilization or contraception, said method comprising contacting a GPR54 polypeptide with a test compound and assaying a biological activity of said GPR54 polypeptide, wherein an alteration of said biological activity, relative to a control not contacted with the said test compound, identifies said test compound as a candidate compound for use in treating infertility, in *in vitro* fertilization or contraception.

6. The method of claim 5, wherein said candidate compound induces a state of hypogonadotropic hypogonadism in a patient.

7. The method of claim 5, wherein said alteration results in a decrease in said biological activity of GPR54.

8. The method of claim 5, wherein said alteration results in an increase in said biological activity of GPR54.

9. A method for treating a reproductive disorder in a mammal, said reproductive disorder being selected from the group consisting of central precocious puberty, polycystic ovarian disease, endometriosis, and uterine fibroids, said method comprising administering to said mammal an effective amount of a compound that decreases a biological activity of a GPR54 polypeptide.

10. The method of claim 9, wherein said compound induces a state of hypogonadotropic hypogonadism in said mammal.

11. A method for treating a reproductive disorder in a mammal, said reproductive disorder being selected from the group consisting of idiopathic hypogonadotropic hypogonadism, amenorrhea, delayed puberty, and polycystic ovarian disease, said method comprising administering to said mammal an effective amount of a compound that increases a biological activity of a GPR54 polypeptide.

12. A method for contraception in a mammal, said method comprising administering to said mammal an effective amount of a compound that alters a biological activity of a GPR54 polypeptide.

13. The method of claim 12, wherein said method further comprises the administration of a steroid to said mammal.

14. A method for treating infertility in a mammal, said method comprising administering to said mammal an effective amount of a compound that alters a biological activity of a GPR54 polypeptide.

15. The method of claim 12 or 14, wherein said compound decreases a biological activity of a GPR54 polypeptide.

16. The method of claim 12 or 14, wherein said compound increases a biological activity of a GPR54 polypeptide.

17. A method of altering the level of a gonadal sex steroid in a mammal with a proliferative disorder, said method comprising contacting a mammal with a compound that decreases a biological activity of a GPR54 polypeptide.

18. The method of claim 17, wherein said compound induces a hypogonadotropic state in said mammal.

19. A method of diagnosing a reproductive disorder in a mammal, said method comprising determining whether said mammal has an alteration in a nucleic acid sequence containing a GPR54 nucleic acid molecule, or a fragment thereof, relative to a control nucleic acid sequence, where said alteration is indicative that said mammal has or has a propensity for developing a reproductive disorder.

20. The method of claim 19, wherein said alteration results in an amino acid change at a position corresponding to amino acid 148, 331, or 399 of a human GPR54 amino acid sequence.

21. A method of diagnosing a reproductive disorder in a mammal, said method comprising determining whether said mammal has an alteration in the expression or biological activity of a GPR54 nucleic acid molecule or GPR54 amino acid molecule, or a fragment thereof, relative to a control nucleic acid sequence or amino acid sequence, where said alteration of expression or biological activity is indicative that said mammal has or has a propensity for developing a reproductive disorder.

22. The method of claim 21, wherein said alteration is an alteration in the expression level of a GPR54 messenger RNA molecule.

23. The method of claim 22, wherein said expression level is determined using reverse transcriptase polymerase chain reaction.

24. The method of claim 21, wherein said alteration is an alteration in a biological activity of a GPR54 polypeptide.

25. The method of claim 24, wherein said biological activity comprises an alteration in the level of inositol phosphate production.

26. The method of claim 19 or 21, wherein said reproductive disorder is selected from the list consisting of delayed puberty, central precocious puberty, idiopathic hypogonadotropic hypogonadism, amenorrhea, and polycystic ovarian disease.

27. The method of any one of claims 9, 11, 12, 14, 19, or 21, wherein said mammal is a human.

28. An isolated GPR54 nucleic acid sequence encoding a polypeptide comprising an amino acid change at a position corresponding to amino acid 148 of a human GPR54 amino acid sequence.

29. The isolated GPR54 nucleic acid sequence of claim 28, wherein said nucleic acid sequence encodes a Serine at said position 148.

30. The isolated GPR54 nucleic acid sequence of claim 29, wherein said nucleic acid sequence comprises a T to C alteration at position 443 of a human GPR54 nucleic acid sequence.

31. An isolated GPR54 nucleic acid sequence encoding a polypeptide comprising an amino acid change at a position corresponding to amino acid 331 of a human GPR54 amino acid sequence.

32. The isolated GPR54 nucleic acid sequence of claim 31, wherein said nucleic acid sequence encodes a stop codon at said position 331.

33. The isolated GPR54 nucleic acid sequence of claim 32, wherein said nucleic acid sequence comprises a C to T alteration at position 991 of a human GPR54 nucleic acid sequence.

34. An isolated GPR54 nucleic acid sequence encoding a polypeptide comprising an amino acid change at a position corresponding to amino acid 399 of a human GPR54 amino acid sequence.

35. The isolated GPR54 nucleic acid sequence of claim 34, wherein said nucleic acid sequence encodes an Arginine at said position 399.

36. The isolated GPR54 nucleic acid sequence of claim 35, wherein said nucleic acid sequence comprises a T to A alteration at position 1195 of a human GPR54 nucleic acid sequence.

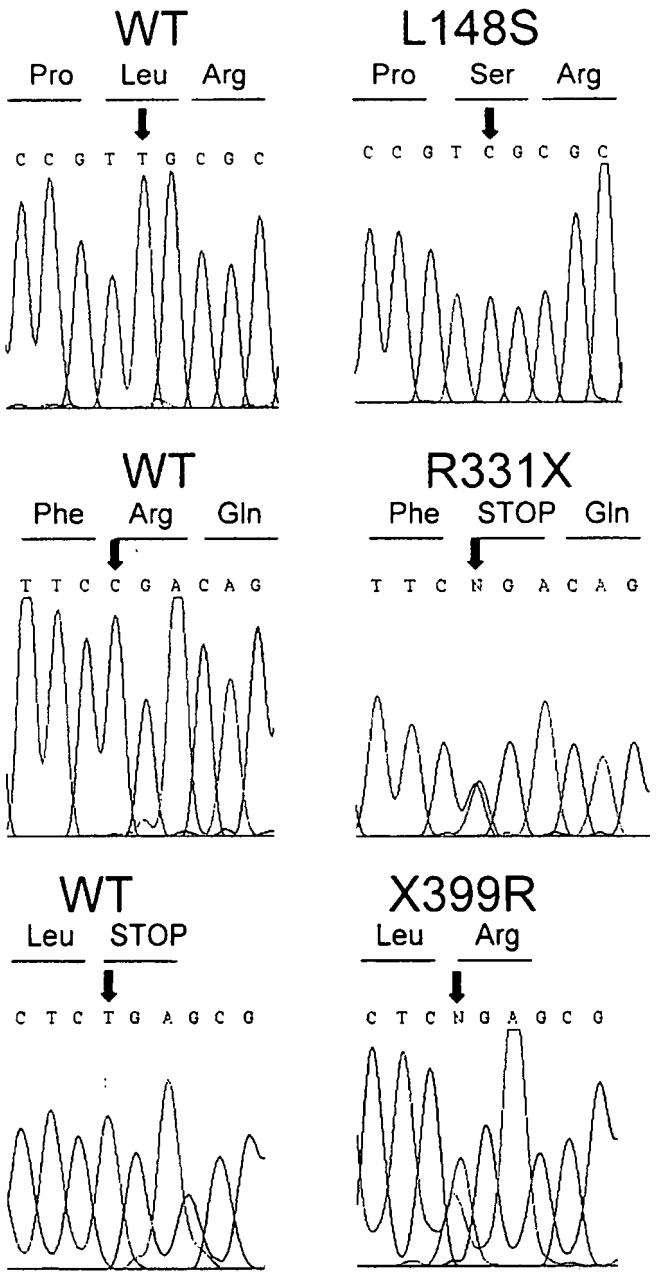
37. A vector comprising the isolated nucleic acid sequence of any one of claims 28, 31, or 34.

38. An isolated cell comprising the vector of claim 37.

39. An isolated polypeptide comprising a human GPR54 amino acid sequence containing an amino acid substitution at position 148, 331, or 399, relative to a polypeptide encoded by GenBank Accession No. AY029541, AF343725, NM_032551, or AY253981.

FIG. 1

A



B

```
Human GPR54      SVDRWYVTVFPLRALHRRTPRL
Mouse Gpr54      SVDRWYVTVFPLRALHRRTPRL
Rat Gpr54        SVDRWYVTVFPLRALHRRTPRL

Human GALR1      SVDRYVAIVHSRRSSSLRVSRN
Human GALR2      SLDRYLAIRYPLHSRELRTPRN
Human GALR3      SVDRYLAVRHPLRSRALRTPRN
Human SSTR1      SVDRYVAVVHPIKAARYRRPTV
Human SSTR2      SIDRYLAVVHPIKSAKWRRPRT
Human SSTR3      SVDRYLAVVHPTRSARWRTAPV
Human SSTR4      SVDRYVAVVHPLRAATYRRPSV
Human SSTR5      SVDRYLAVVHPLSSARWRRPRV
7tm consensus    SIDRYLAIVHPLRYRRIRTPRR
```

C

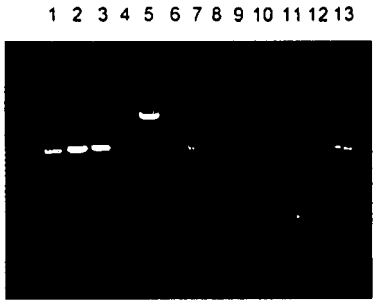
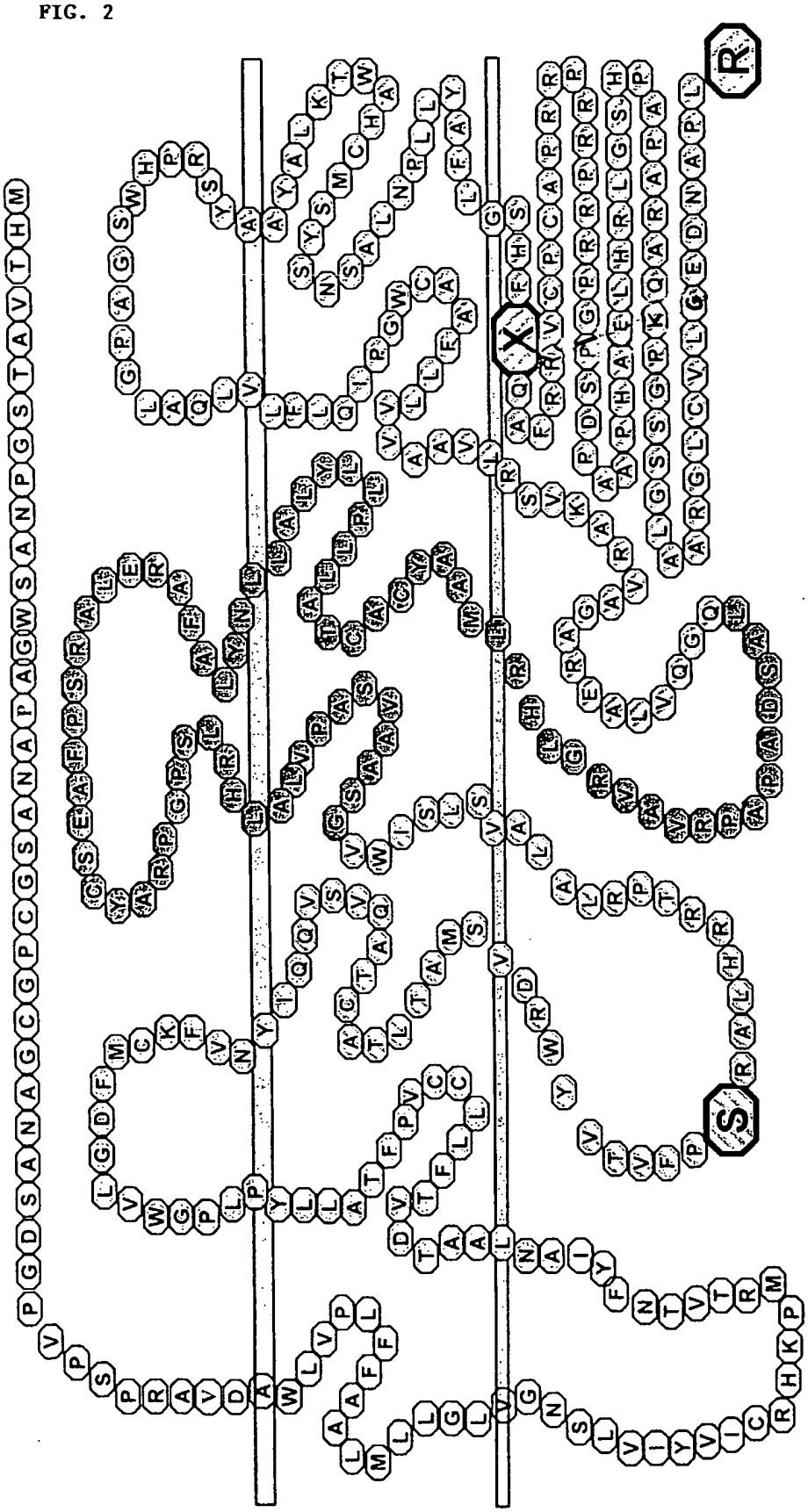


FIG. 2



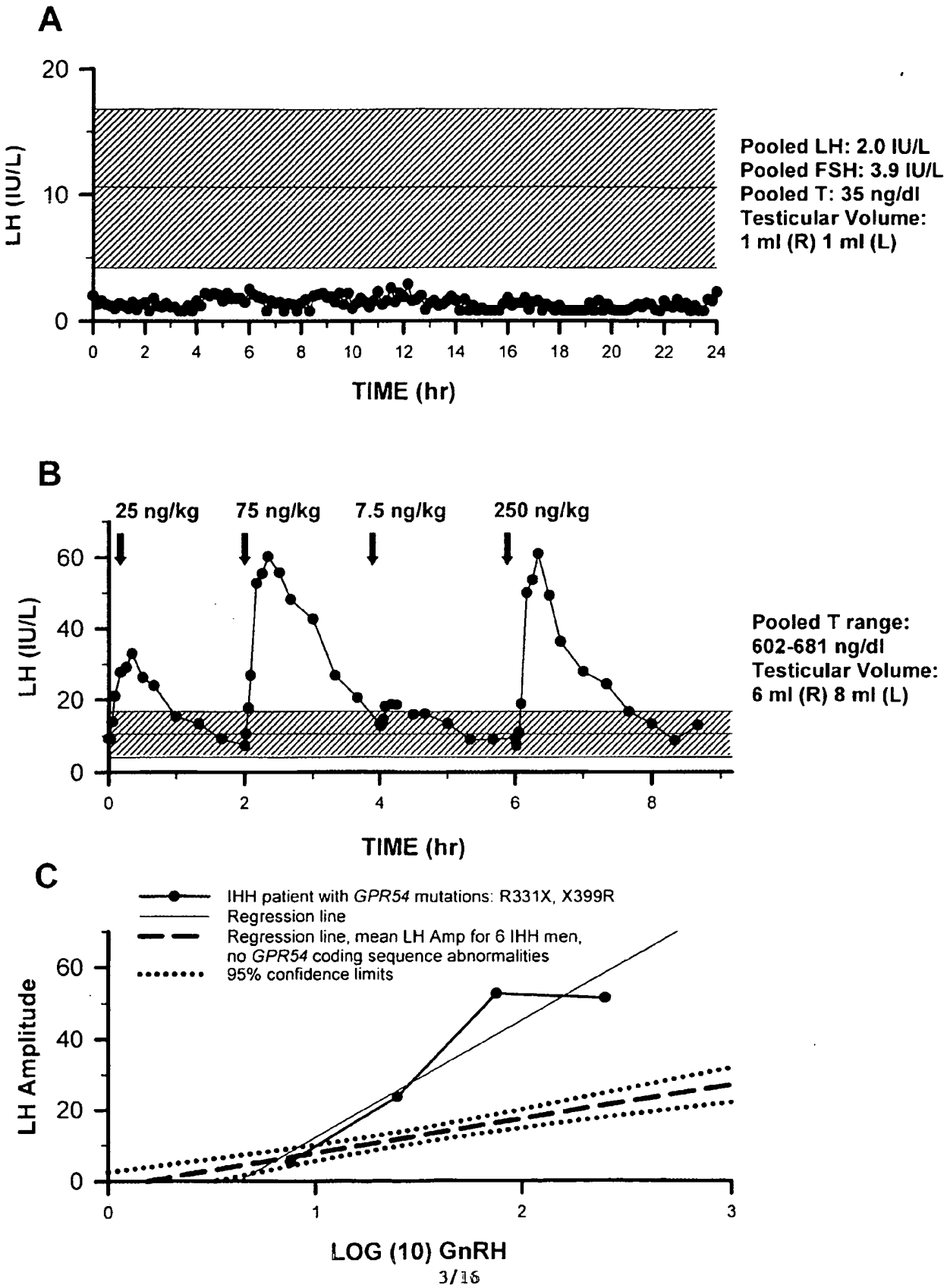

A
LH (IU/L)
TIME (hr)
Pooled LH: 2.0 IU/L
Pooled FSH: 3.9 IU/L
Pooled T: 35 ng/dl
Testicular Volume:
1 ml (R) 1 ml (L)
B
25 ng/kg
75 ng/kg
7.5 ng/kg
250 ng/kg
LH (IU/L)
TIME (hr)
Pooled T range:
602-681 ng/dl
Testicular Volume:
6 ml (R) 8 ml (L)
C
IHH patient with GPR54 mutations: R331X, X399R
Regression line
Regression line, mean LH Amp for 6 IHH men,
no GPR54 coding sequence abnormalities
95% confidence limits
LH Amplitude
LOG (10) GnRH

FIG. 4

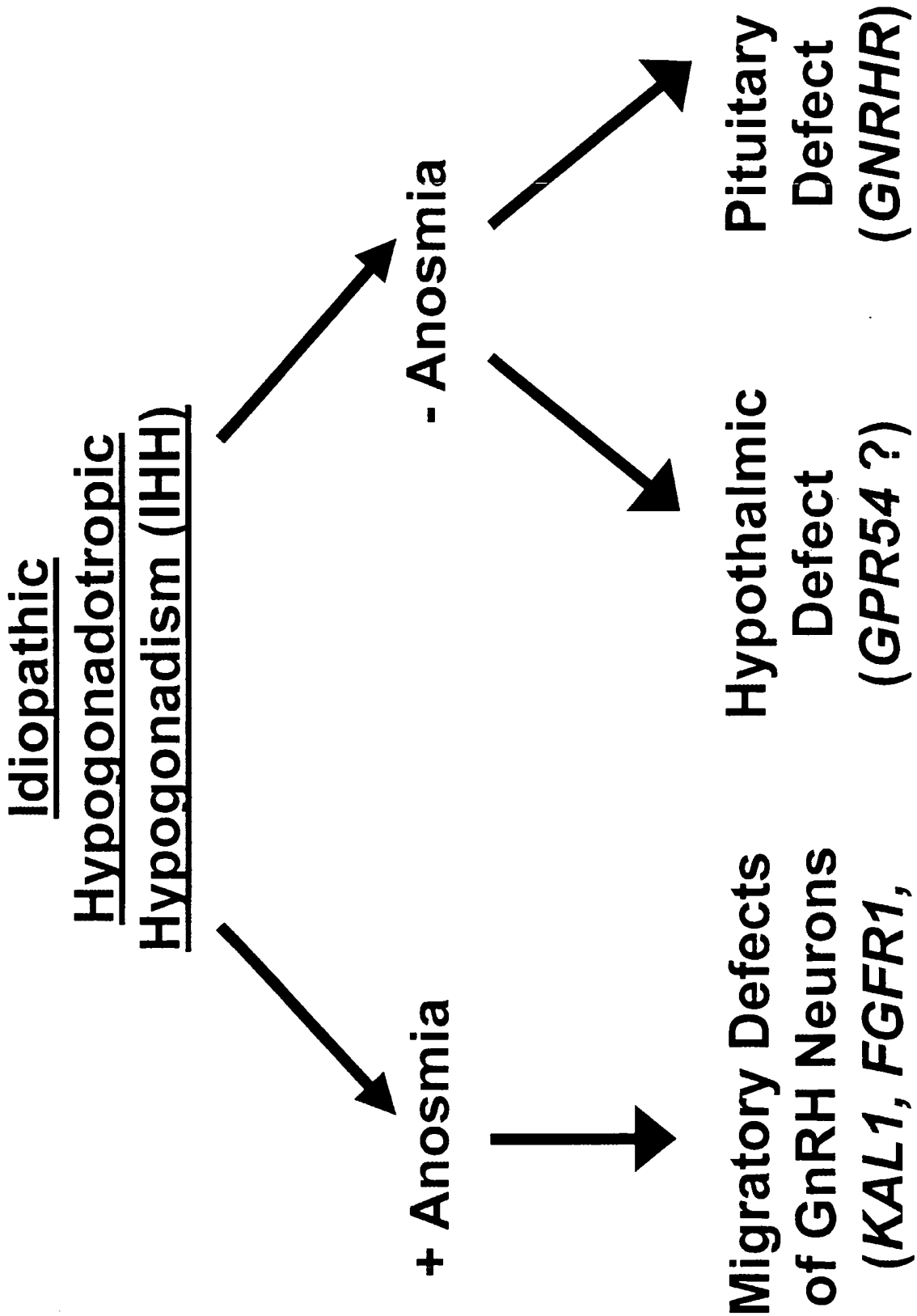

Idiopathic
Hypogonadotropic
Hypogonadism (IHH)
- Anosmia
+ Anosmia
Pituitary
Defect
(GNRHR)
Hypothalmic
Defect
(GPR54 ?)
Migratory Defects
of GnRH Neurons
(KAL1, FGFR1,
GPR54 ?)

FIG. 5

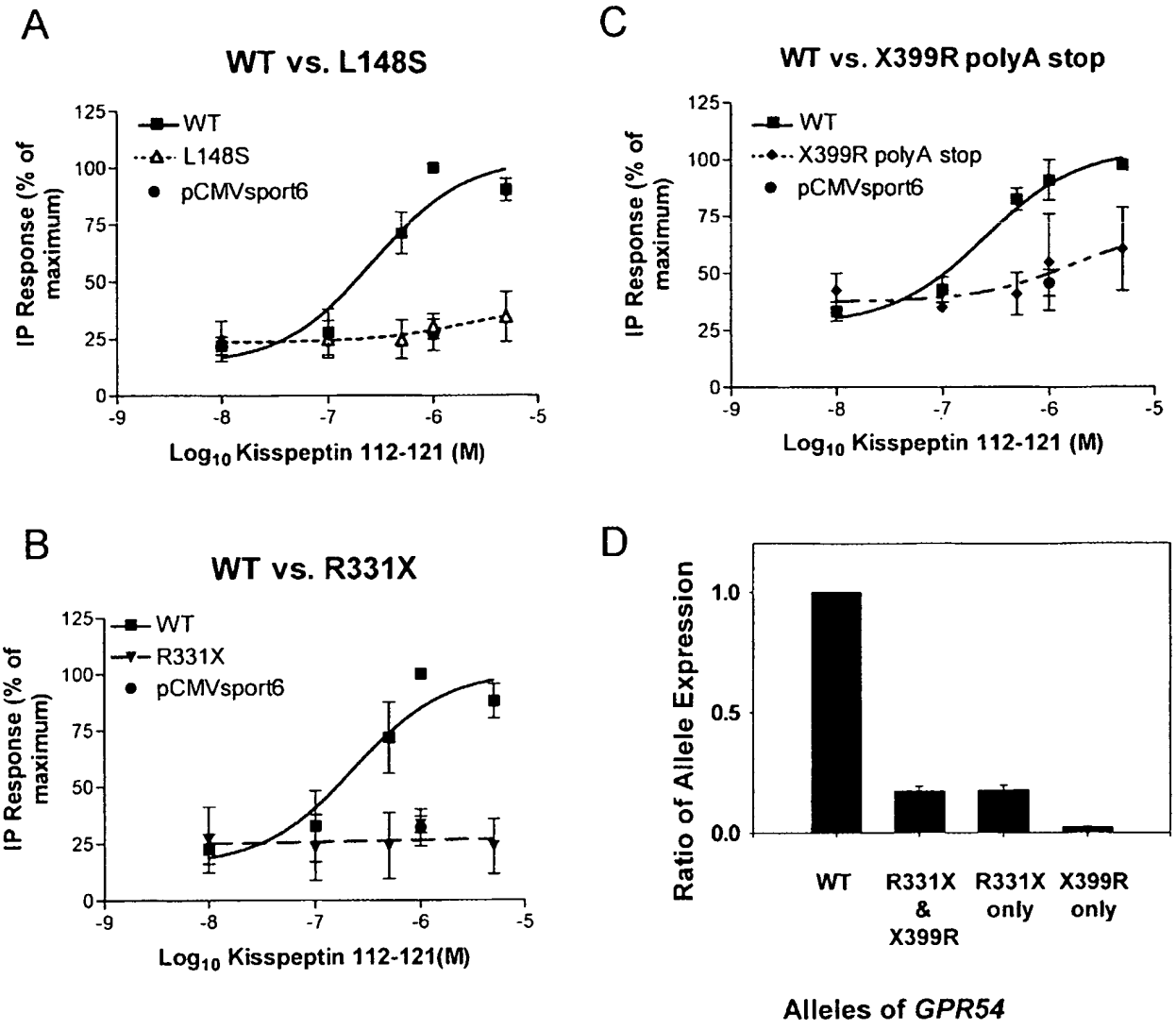

A
WT vs. L148S
WT
L148S
pCMVsport6
IP Response (% of maximum)
Log10 Kisspeptin 112-121 (M)
B
WT vs. R331X
WT
R331X
pCMVsport6
IP Response (% of maximum)
Log10 Kisspeptin 112-121(M)
C
WT vs. X399R polyA stop
WT
X399R polyA stop
pCMVsport6
IP Response (% of maximum)
Log10 Kisspeptin 112-121 (M)
D
Ratio of Allele Expression
WT
R331X & X399R
R331X only
X399R only
Alleles of GPR54

FIG. 6A

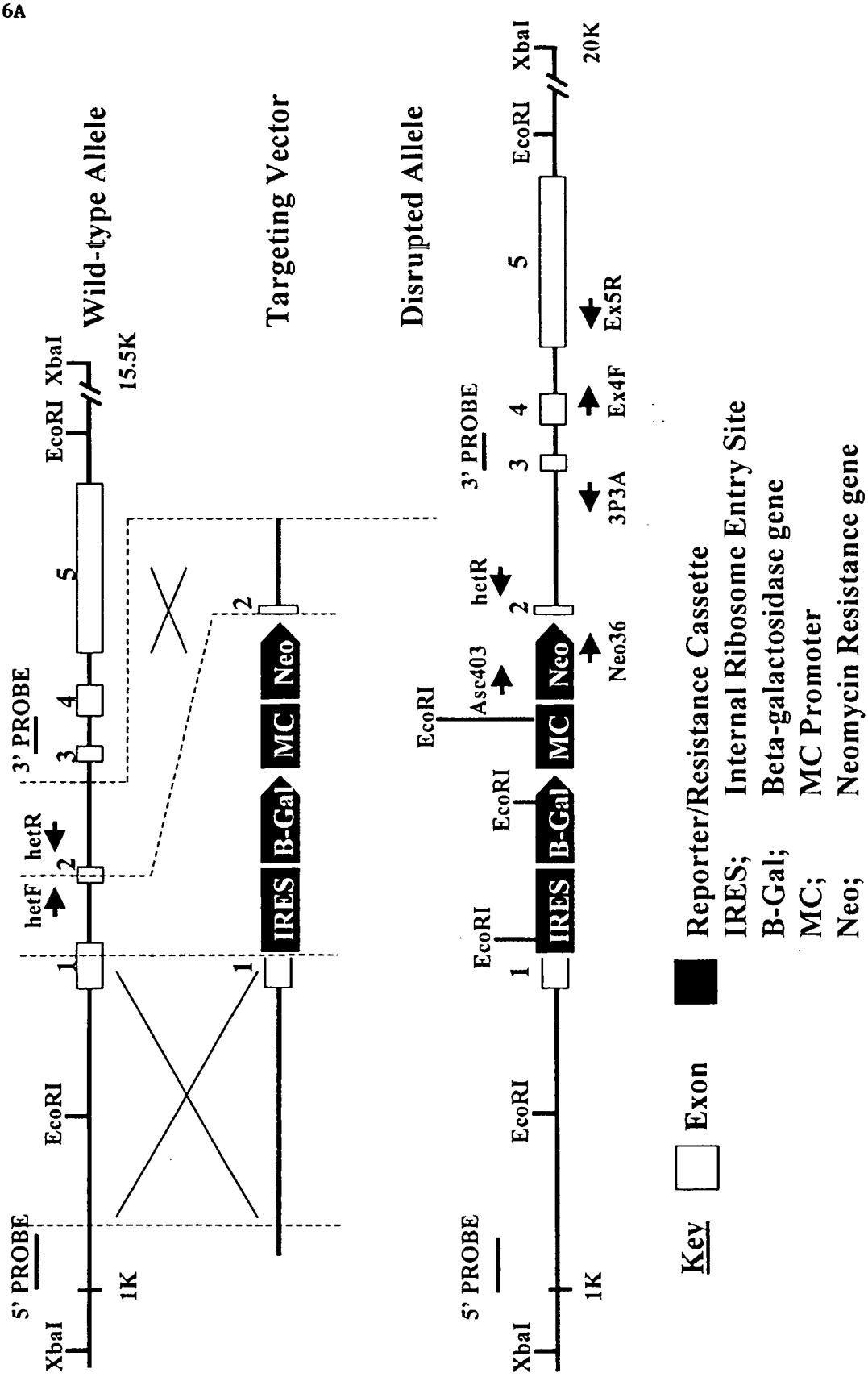

Wild-type Allele
Targeting Vector
Disrupted Allele
XbaI
EcoRI
XbaI
15.5K
5' PROBE
3' PROBE
hetF
hetR
EcoRI
IRES
B-Gal
MC
Neo
Asc403
Neo36
3P3A
Ex4F
Ex5R
1K
20K
Key
Exon
Reporter/Resistance Cassette
IRES; Internal Ribosome Entry Site
B-Gal; Beta-galactosidase gene
MC; MC Promoter
Neo; Neomycin Resistance gene

FIG. 6B

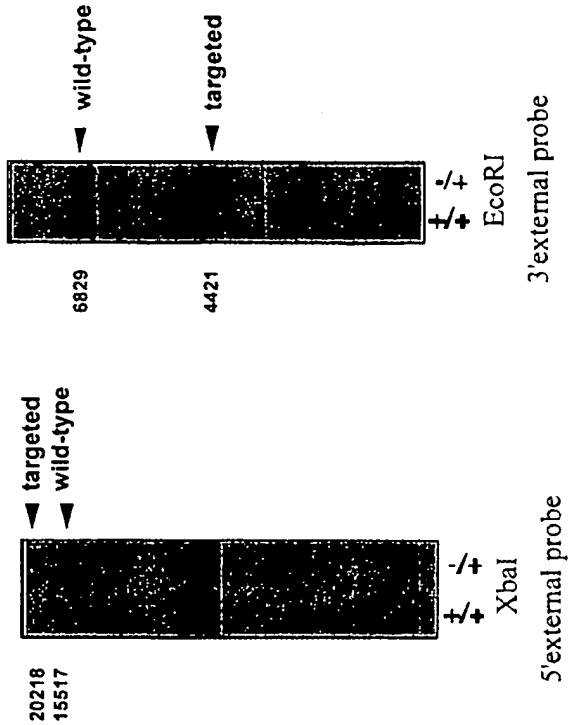
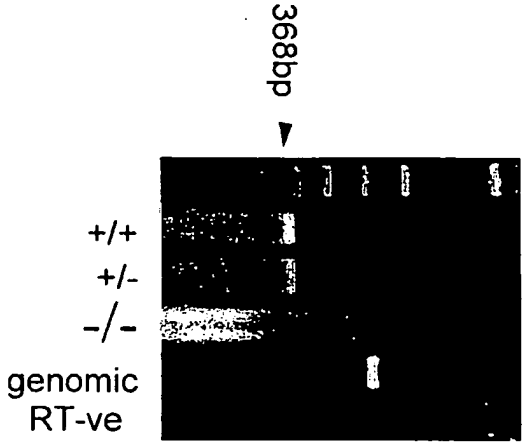

wild-type
targeted
-/+
+/+
EcoRI
3'external probe
6829
4421
targeted
wild-type
-/+
+/+
XbaI
5'external probe
20218
15517

FIG. 6C



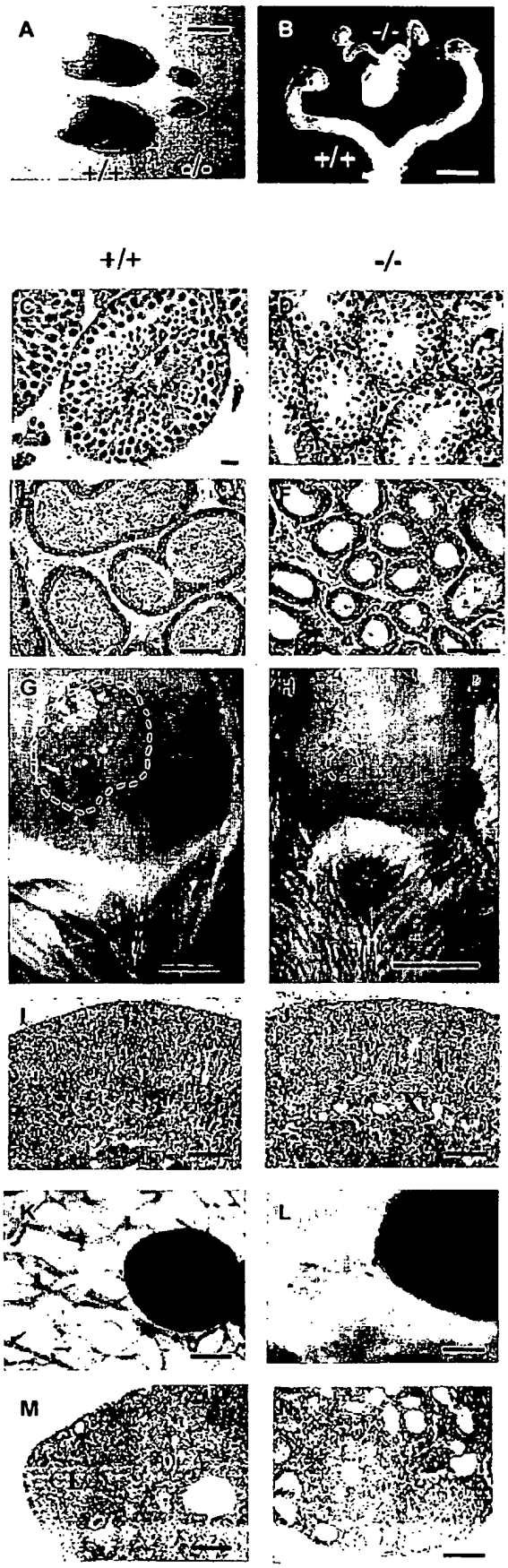

A
+/+
d/o
B
-/-
+/+
+/+
-/-
C
D
E
F
G
H
I
J
K
L
M

FIG. 8

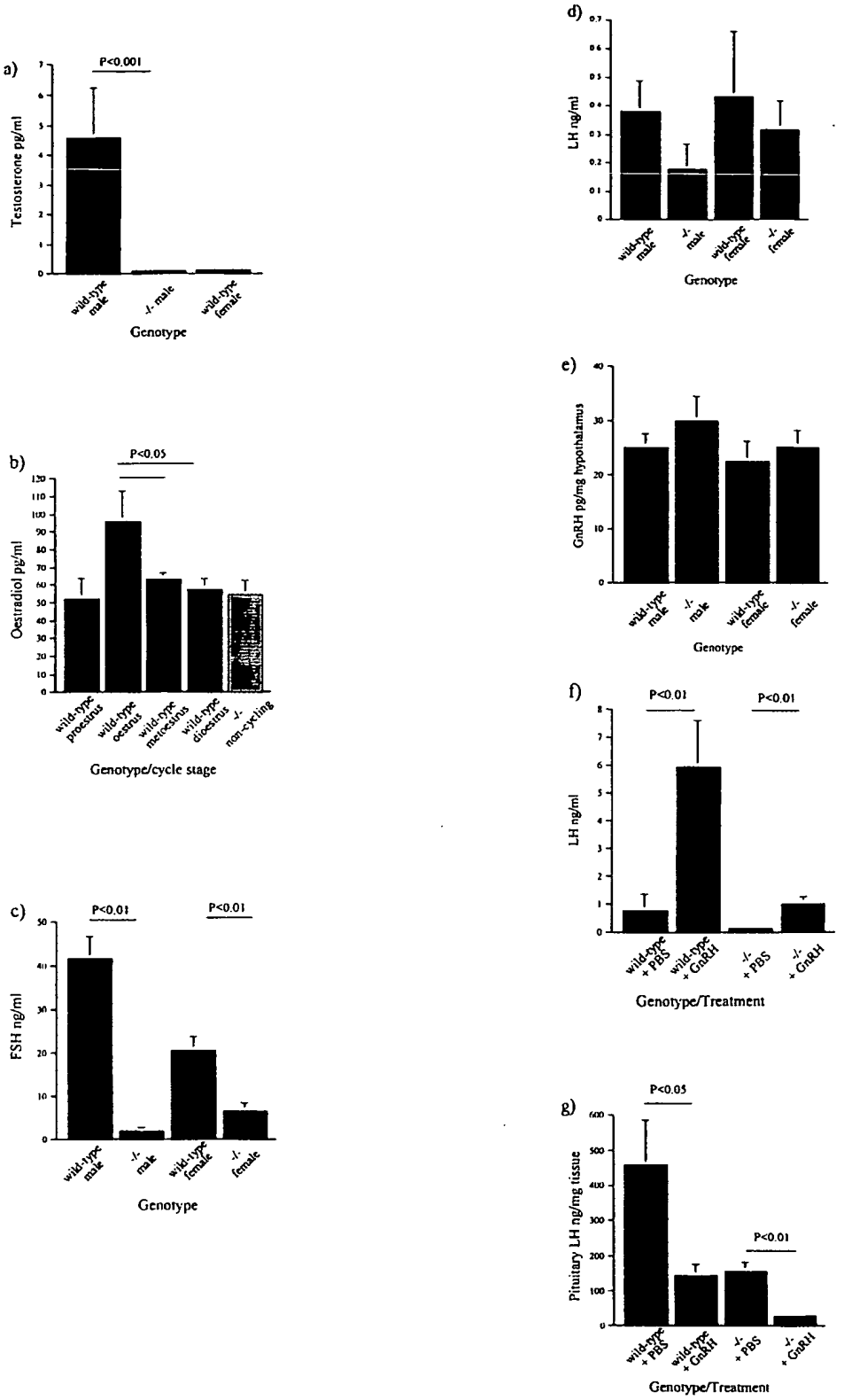

a)
P<0.001
Testosterone pg/ml
wild-type male
-/- male
wild-type female
Genotype
b)
P<0.05
Oestradiol pg/ml
wild-type proestrus
wild-type oestrus
wild-type metoestrus
wild-type dioestrus
-/- non-cycling
Genotype/cycle stage
c)
P<0.01
P<0.01
FSH ng/ml
wild-type male
-/- male
wild-type female
-/- female
Genotype
d)
LH ng/ml
wild-type male
-/- male
wild-type female
-/- female
Genotype
e)
GnRH pg/mg hypothalamus
wild-type male
-/- male
wild-type female
-/- female
Genotype
f)
P<0.01
P<0.01
LH ng/ml
wild-type + PBS
wild-type + GnRH
-/- + PBS
-/- + GnRH
Genotype/Treatment
g)
P<0.05
P<0.01
Pituitary LH ng/mg tissue
wild-type + PBS
wild-type + GnRH
-/- + PBS
-/- + GnRH
Genotype/Treatment

FIG. 9A

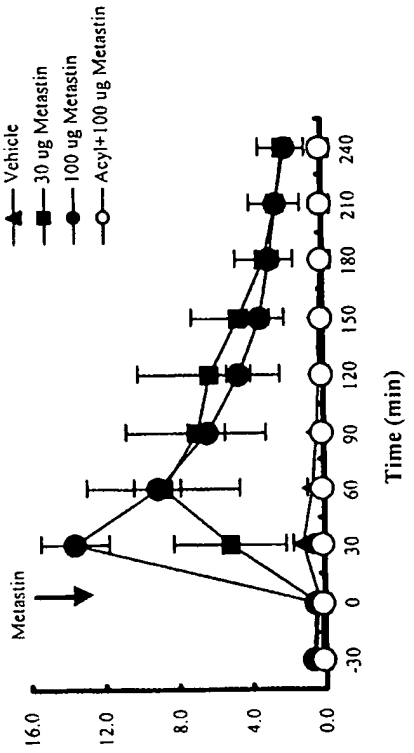

Vehicle
30 ug Metastin
100 ug Metastin
Acyl+100 ug Metastin
Metastin
16.0
12.0
8.0
4.0
0.0
-30
0
30
60
90
120
150
180
210
240
Time (min)

FIG. 9B

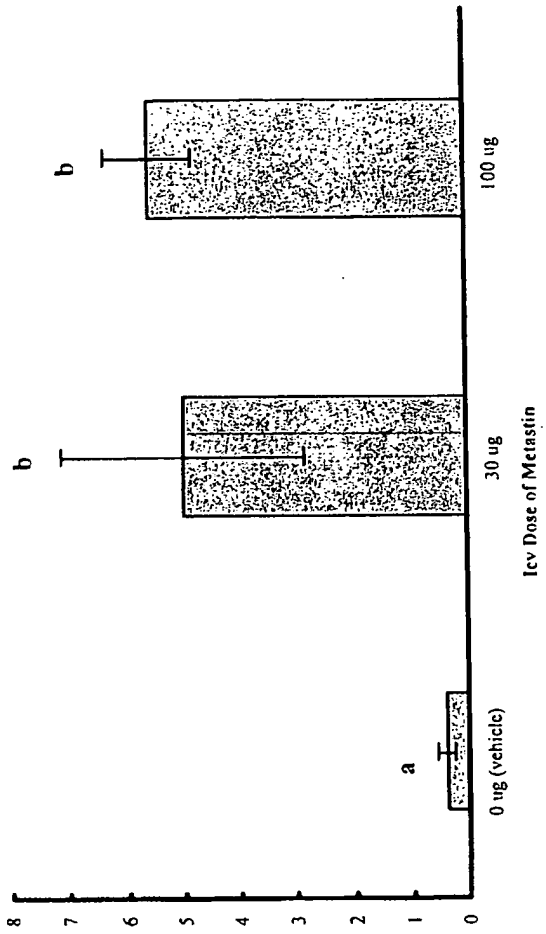


FIG. 10

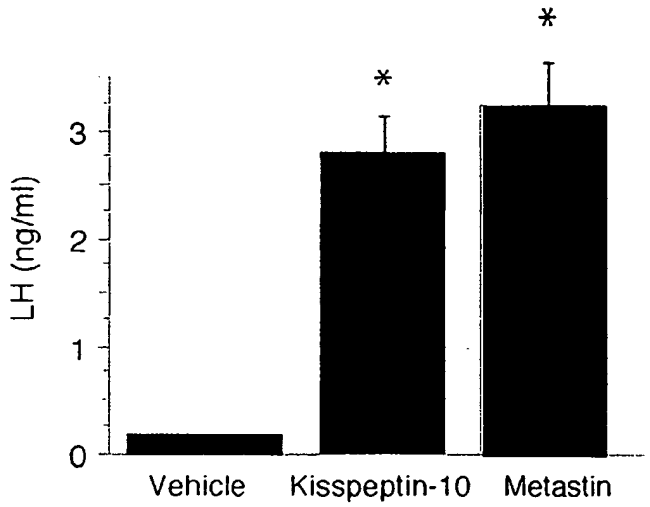


FIG. 11

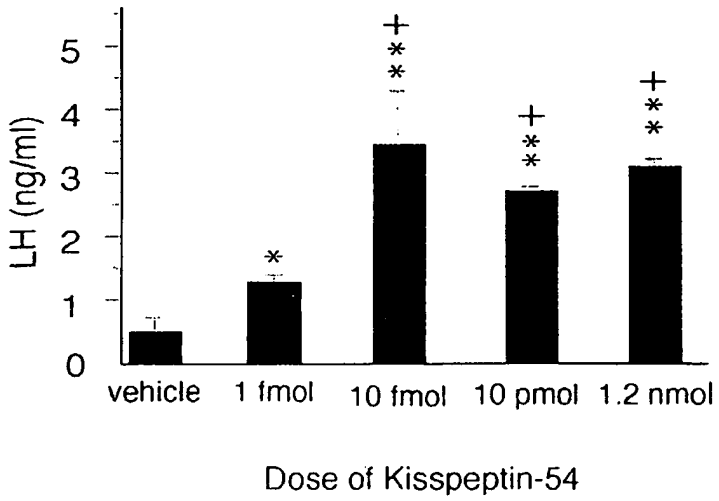

LH (ng/ml)
0
1
2
3
4
5
vehicle
1 fmol
10 fmol
10 pmol
1.2 nmol
Dose of Kisspeptin-54

FIG. 12A

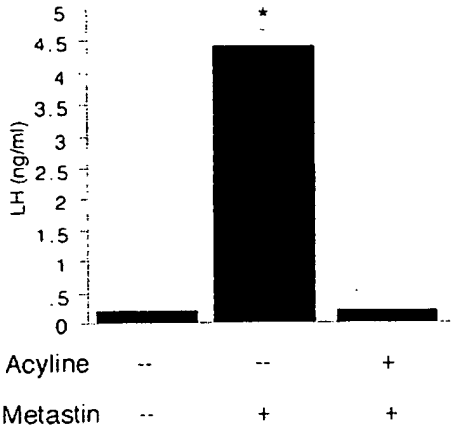


FIG. 12B

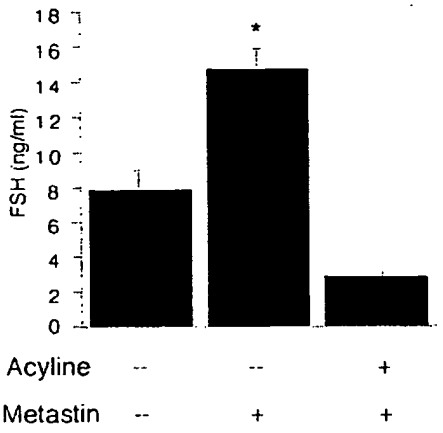


FIG. 13